IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Before the Board of Patent Appeals and Interferences

In re Patent Application of

LI et al.

Serial No. 09/145,180

Filed: September 1, 1998

Title: OXO-SUBSTITUTED COMPOUNDS, PROCESS OF MAKING, AND COMPOSITIONS AND METHODS FOR INHIBITING PARP ACTIVITY

Atty Dkt. 2824-9
C# M#

Group Art Unit: 1617

Examiner: Wang, S.

Date: May 27, 2003

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

☒ **Correspondence Address Indication Form Attached.**

☐ **NOTICE OF APPEAL**
Applicant hereby appeals to the Board of Appeals from the decision dated _____ of the Examiner twice/finally rejecting claims _____ ($ 320.00) $

☒ An appeal **BRIEF** is attached in triplicate in the pending appeal of the above-identified application ($ 320.00) $ 320.00

☐ Credit for fees paid in prior appeal without decision on merits -$ ( )

☐ A reply brief is attached in triplicate under Rule 193(b) **(no fee)**

☐ Petition is hereby made to extend the current due date so as to cover the filing date of this paper and attachment(s) **($110.00/1 month**; $410.00/2 months; **$930.00/3 months**; $1450.00/4 months) $ 1450.00

SUBTOTAL $ 1770.00

☒ Applicant claims "Small entity" status, enter ½ of subtotal and subtract -$(885.00)
☐ "Small entity" statement attached.

SUBTOTAL $ 885.00

Less month extension previously paid on -$(0.00)

TOTAL FEE ENCLOSED $ 885.00

Any future submission requiring an extension of time is hereby stated to include a petition for such time extension. The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our **Account No. 14-1140.** A duplicate copy of this sheet is attached.

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By Atty: B. J. Sadoff, Reg. No. 36,663

Signature: B. Sadoff

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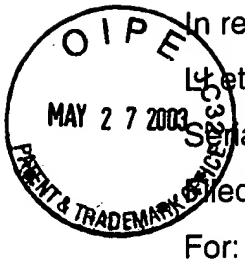
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES



In re Patent Application of

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Atty. Ref.: 2824-9

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Examiner: Wang, S.

For: OXO-SUBSTITUTED COMPOUNDS, PROCESS OF MAKING, AND COMPOSITIONS AND METHODS FOR INHIBITING PARP ACTIVITY

* *

Tuesday, May 29, 2003

Mail Stop Appeal Brief-Patents

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

Appellants hereby appeals the Final Rejection of independent claims 184 and 210 and dependent claims 185-192, 207, 209, 211 -213 and 216, in the Office Action dated May 24, 2002, Paper No. 26, and submits the present Appeal Brief, in triplicate, pursuant to Rule 192.

(1) Real party in interest

The real party in interest is Guilford Pharmaceuticals, Inc., 6611 Tributary Street, Baltimore, MD 21224, by way of an Assignment from the applicants,

recorded in the U.S. Patent and Trademark Office on September 1, 1998, at Reel 9447, Frame 0851.

(2) Related appeals and interferences

The appellant, the undersigned, and the assignee are not aware of any related appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

(3) Status of the claims

Originally-filed claims 1-183 were canceled, and claims 184-233 were added, in an Amendment filed August 24, 2000. Claims 217, 224, 227 and 229 were amended in an Amendment filed June 29, 2001, which was not originally entered (see, Advisory Action dated July 19, 2001 (Paper No. 19)), but was entered upon filing of a CPA on August 30, 2001.

Claims 184-233 are pending and are attached as Appendix A.

Claims 193-195, 197-206, 208, 214, 215 and 217-233 have been withdrawn from consideration as being drawn to a non-elected invention. Basis for withdrawing claims 193-195 197-206, 208, 214, 215 and 217-233 from examination is not clear as all the

pending claims are directed to the originally elected "method of using compounds" (Group II), as opposed to "compounds and pharmaceutical composition" (original Group I of Paper No. 10) or "method of making compounds" (original Group III of Paper No. 10). The Examiner has allowed the elected species (i.e., 5(*H*)2-nitro-10-aminophenanthridin-6-one, see, below and claim 196) and not provided a basis for withdrawing claims 193-195, 197-206, 208, 214, 215, and 217-233 from consderation, or further defined a subgenus which is the subject of the examination. See, page 1 of the Office Action dated October 30, 2000 (Paper No. 16) which only indicates that claims 193-195, 197-206, 208, 214, 215, 218, 219, 221-223, 226 and 231-233 are withdrawn from consideration. In the final rejection of May 24, 2002 (Paper No. 26), claims 193-195, 197-206, 208, 214, 215 and 217-233 have been withdrawn from consideration, again without justification or basis or explanation. The Examiner is requested to define, with particularity, the alleged separately patentable subject matter and the scope of the present search, for clarity of the record and convenience of the Board.

Claim 196 has been both rejected as allegedly being obvious and objected to as being dependent upon a rejected base claim, "but would be allowable if rewritten in independent form including all the limitations of the base claim [i.e., independent claim 184] and any intervening claims." See, page 2 of the Office Action dated May 24, 2002 (Paper No. 26). As each claim is examined independently, claim 196 is treated herein as having been allowed and not the subject of the present appeal. Should the Board

affirm the Examiner, the Board is requested to return the application to the Examiner for cancellation of any rejected and non-elected subject matter and amendment of claim 196 to place the claim in condition for allowance.

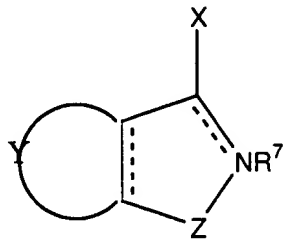
At a minimum, claims 185-192, 207, 209, 211-213 and 216 are the subject of the present appeal as at least these claims have clearly been finally rejected. A copy of all the pending claims 184-233 is attached as Appendix A for completeness.

(4) Status of amendments

No amendments have been filed in response to the final rejection (Paper No. 26). A Response to the final rejection was filed September 24, 2002. The Response of September 24, 2002 was considered.

(5) Summary of the invention

The claimed invention is directed to a method of inhibiting PARP activity comprising administering a compound of formula I containing at least one ring nitrogen:



and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*, or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, where X is double-bonded oxygen or -OH; R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring; where said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent; and

when the Y and Z rings are phenyl and X is double bonded oxygen, at least one of the Y and Z rings contains at least one said substituent. See, page 24, line 25 - page 27, line 2, page 57, line 25 - page 67, line 5, and independent claim 184.

In another embodiment of the invention, X is double- bonded oxygen. See, page 24, line 27 and dependent claim 185.

In another embodiment of the invention, Y has at least one site of unsaturation. See, page 25, lines 20-21 and dependent claim 186.

In another embodiment of the invention, Y represents the atoms necessary to form a fused benzene or naphthalene ring. See, page 25, lines 11-14 and dependent claim 187.

In another embodiment of the invention, Y is substituted with at least one non-hydrogen, non-interfering substituent. See, page 25, lines 20-24 and dependent claim 188.

Support for the recitations of dependent claims 189 and 216 may be found, for example, at page 25, line 25 to page 26, line 29 of the specification.

Support for the recitations of dependent claim 190 may be found, for example, on page 27, line 19 to page 28, line 35 of the specification.

Support for the recitation of dependent claims 191 and 192 may be found, for example, at page 23, lines 29-37 of the specification, and originally-filed claims 48 and 49.

Support for the recitations of dependent claim 207 may be found, for example, page 54, line 9 to page 56, line 14 of the specification, and originally-filed claim 73.

Support for the recitations of dependent claim 209 may be found, for example, in originally-filed claim 77, and the corresponding portion of the disclosure.

Support for the recitations of dependent claim 210 may be found, for example, on page 57, lines 25-39, as well as the indicated support for claim 84.

Support for the recitations of dependent claim 211 may be found, for example, at page 21, line 1 of the specification.

In another embodiment of the invention, the neuronal activity of the claims includes stimulation of damaged neurons, promotion of neuronal regeneration,

prevention of neurodegeneration, and treatment of a neurological disorder. See, page 57, lines 29 to 32, and dependent claim 212.

Support for the recitations of dependent claim 213 may be found at page 57, lines 35-39 of the specification.

(6) Issues

The following issue is presented for appeal:

Whether the method of claims 184-192, 207, 209-213 and 216 would have been obvious at the time of the invention was made to a person having ordinary skill in the art as being unpatentable over Weltin et al. (Oncology Research, Vol. 6, No. 9, pp. 399-403 (1994)) in view of Banasik et al. (Journal of Biological Chemistry, Vol. 267, No. 3, pp.1569-1575 (1992)), Suto et al. (Anti-Cancer Drug Design No. 7, pp. 107-117, (1991)) and Endres et al. (Journal of Cerebral Blood Flow and Metabolism, No. 17, pp. 1143-1151, (1997)).

(7) Grouping of the claims

Claims 184-192, 207, 209-213 and 216 stand or fall together.

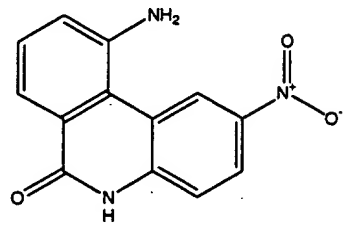
(8) Argument

The rejection of claims 184-192, 207, 209-213 and 216 under 35 U.S.C. § 103 over Weltin et al. (Oncology Research, Vol. 6, No. 9, pp. 399-403 (1994)) in view of Banasik et al. (Journal of Biological Chemistry, Vol. 267, No. 3, pp.1569-1575 (1992)), Suto et al. (Anti-Cancer Drug Design No. 7, pp. 107-117, (1991)) and Endres et al. (Journal of Cerebral Blood Flow and Metabolism, No. 17, pp. 1143-1151, (1997)) should be reversed.

A copy of the cited art is provided herewith as Appendix B for the convenience of the Board.

The appellants note a species election was made in the Response of March 15, 2000, to the compound of claim 125 (now recited in pending claim 196, which has been indicated as being allowed), with a mode of delivery of sterile solution, preferably for intravenous administration, and an indication of treating ischemia/reperfusion. The Examiner withdrawn claims 193-195, 197-206, 208, 214, 215, 218-291, 221-223, 226 and 231-233 from consideration as allegedly being drawn to a non-elected species where there is allegedly no allowable generic or linking claim. As noted above, the Examiner is not believed to have clearly defined the scope of the examination or defined the allegedly separately patentable invention. For at least clarity of the record and the convenience of the Board, the Examiner is requested to do so in the Examiner's Answer.

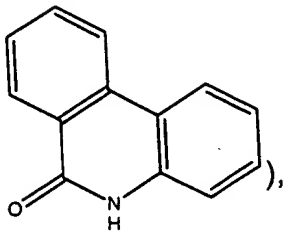
The Appellants note, for the convenience of the Board, that the elected (and allowed) species has the following structure:



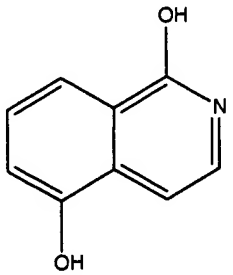
5(H)2-nitro-10-aminophenanthridin-6-one.

The Section 103 rejection should be reversed as the combination of cited art fails to establish a *prima facie* case of obviousness. Consideration of the following in this regard is requested.

The Board will appreciate that to establish a *prima facie* case of obviousness, the cited art, coupled with the knowledge generally available in the art at the time of the invention, must contain some suggestion or incentive that would have motivated the ordinarily skilled artisan to modify a reference or to combine references, to make the claimed invention. See, *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596 (Fed.Cir. 1988); *In re Skinner*, 2 U.S.P.Q.2d 1788, 1790 (Bd. Pat. App. & Int. 1986) (copies attached hereto as Appendix C).

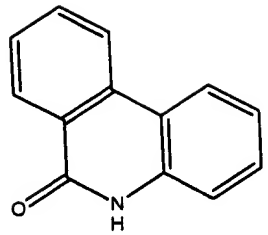
The Examiner is understood to have asserted that the primary reference, Weltin, teaches that 6(5)-phenanthridinone (i.e., ), 6(5H)-phenanthridinone is a potent inhibitor of PARP, and that one of the secondary reference, Endres, teaches a method of treating ischemia by inhibition of PARP activity. The Examiner asserts that one of ordinary skill in the art would have reasonably expected to optimize PARP inhibiting activity of 6(5H) - phenanthidinone "by adding amino and/or nitro group[s] to 6(5H)-phenanthridinone", allegedly based on Banasik's teaching of a 2-nitro substituted 6(5H)- phenanthridinone. See, pages 2-3 of Paper No. 26.

In fact, Weltin discusses three structurally distinct compounds that are taught to provide PARP inhibiting activity, i.e., 1, 5-dihydroxyisoquinoline;



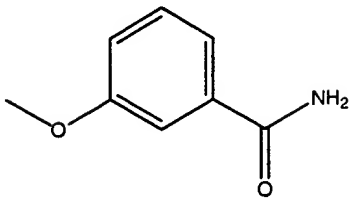
1,5-dihydroxyisoquinoline

6(5H)-phenanthridinone



6(5H)-phenanthridinone

and 3-methoxybenzamide;

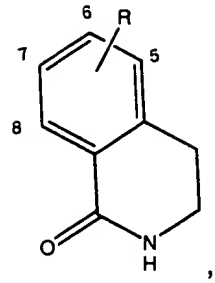


3-methoxybenzamide

Only one of these noted structures of Weltin (i.e., the 6(5H) phenanthridinone selected by the Examiner) is a phenanthridinone. Weltin, including all 3 different types of structures, must be considered as a whole. The Examiner's selection of and focus on 6(5H)-phenanthridinone is an inappropriate use of hindsight. Weltin does not provide any motivation to select the 6(5H)-phenanthridinone over the other structurally unrelated compounds.

The secondary reference, Banasik, discloses 76 different compounds, only two of which are phenanthrindinones. Absent the appellants disclosure, there was no motivation in Banasik to focus on phenanthridinones.

The Examiner's other secondary reference, i.e., Suto, discloses that substituted isoquinolines of the following structure:



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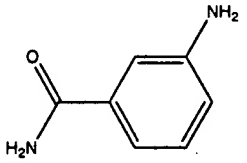
would have a wide range of activity, some of which have only minimal activity (i.e., IC_{50}= 120 uM).

It is unclear from the cited art how or why one of ordinary skill would have have altered one of the structures of Weltin (i.e., selecting to alter the phenanthridinone) in view of Suto with or without Banasik. In fact, the appellants believe one of ordinary skill may have been motivated to alter, if anything, the 1,5-dihydroxyquinoline of Weltin from the teachings of substituted isoquinolines of Suto, as opposed to the 6(5H)-phenanthridinones.

Finally, Endres only relates to the use of 3-aminobenzamide which has the following structure:



3-aminobenzamide .

The combination, of these references would, at best, only provide an invitation to further experimentation, considering the wide range of chemical species, rather than making the presently claimed invention obvious. The Section 103 rejection should be reversed.

As a further distinction over the cited art, the appellants noted that Endres utilizes 3-aminobenzamide ("3-AB") in analyzing the function of PARP. 3-AB is fundamentally different from the compounds of the instant invention with respect to structure. 3-AB employs a free primary amine ($-NH_2$), as opposed to a secondary amine (-NH-) that is part of a ring structure.

In Suto, the Examiner argues that a method of optimization of PARP activity of isoquinoline derivatives is taught using a substituent at the 5 position gives optimal results. The cited art fails however to suggest or motivate one of ordinary skill to modify the compounds of the cited art. The isoquinoline compounds in Suto have very few substitutions at the 5, 6, 7, and 8 positions, whereas the substantial number of substitutions on the compounds of the presently claimed invention reside on the "other side" of the isoquinoline core. As noted above, there was no motivation in the cited references to combine the same and the combination has been made through an inappropriate use of hindsight.

Finally, the cited art fails to provide a reasonable expectation of success, determined from the vantage point of the skilled artisan at the time the invention was made. See *Amgen, Inc v. Chugai Pharm. Co.*, 927 F.2d 1200, 1209, 18 U.S.P.Q.2d 1016, 1023 (Fed. Cir. 1991) (copy attached with Appendix C).

Suto discloses an amino substituted isoquinoline and Banasik discloses a 2-nitro substituted 6(5H)-phenanthridinone, however the appellants submit that their combination would have been obvious. Specifically, the active component of these

PARP inhibiting compounds that binds to the enzyme is the –CO-NH- group, and, in the cited isoquinolines, that group is contained within a saturated ring structure which provides the entire molecule with a non-planar conformation. However, the 6(5H)-phenanthridinone-type compounds have a planar conformation in which the –CO-NH- group is contained within an unsaturated ring structure. Thus, these two different conformations would have been expected to have had varying impact on the activity of the compound.

The claims, as a whole, are submitted to be patentable over the cited art and reversal of the Section 103 rejection is requested.

In conclusion it is believed that the application is in clear condition for allowance; therefore, early reversal of the Final Rejection and passage of the subject application to issue are earnestly solicited.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

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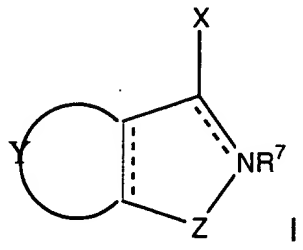
APPENDIX A
PENDING CLAIMS

184. A method of inhibiting PARP activity comprising administering a compound of formula I containing at least one ring nitrogen:



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and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C{=}CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

APPENDIX A
PENDING CLAIMS

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent; and

when the Y and Z rings are phenyl and X is double bonded oxygen, at least one of the Y and Z rings contains at least one said substituent.

185. The method of claim 184, wherein X is double- bonded oxygen.

186. The method of claim 184, wherein Y has at least one site of unsaturation.

187. The method of claim 184, wherein Y represents the atoms necessary to form a fused benzene or naphthalene ring.

188. The method of claim 184, wherein Y is substituted with at least one non-hydrogen, non-interfering substituent.

189. The method of claim 184 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino,

APPENDIX A
PENDING CLAIMS

amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

190. The method of claim 184, wherein:

X is double bonded-oxygen;

Y is a fused benzene ring optionally substituted with a nitro group, an alkyl, an amino group, a halo group, a hydroxy, or a nitroso group; and

Z is $-R^6C=CR^3-$ where R^3 and R^6, taken together, form a fused phenyl ring substituted with a substituent selected from the group consisting of a halo group, a nitro group, an alkyl, an amino group, a hydroxy, or a nitroso group.

191. The method of claim 184, wherein said compound has an IC_{50} for inhibiting poly(ADP-ribose) polymerase *in vitro* of 100 μM or lower.

192. The method of claim 184, wherein said compound has an IC_{50} for inhibiting poly(ADP-ribose) polymerase *in vitro* of 25 μM or lower.

193. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-methylphenanthridin-6-one.

APPENDIX A
PENDING CLAIMS

194. The method of claim 184, wherein said compound is 5(*H*)2-nitro-10-methylphenanthridin-6-one.

195. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-aminophenanthridin-6-one.

196. The method of claim 184, wherein said compound is 5(*H*)2-nitro-10-aminophenanthridin-6-one.

197. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-nitrophenanthridin-6-one.

198. The method of claim 184, wherein said compound is 5(*H*)2,10-dinitrophenanthridin-6-one.

199. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-hydroxyphenanthridin-6-one.

200. The method of claim 184, wherein said compound is 5(*H*)2-nitro-10-hydroxyphenanthridin-6-one.

APPENDIX A
PENDING CLAIMS

201. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-bromophenanthridin-6-one.

202. The method of claim 184, wherein said compound is 5(*H*)2-nitro-10-bromophenanthridin-6-one.

203. The method of claim 184, wherein said compound is 5(*H*)2-chloro-10-nitrosophenanthridin-6-one.

204. The method of claim 184, wherein said compound is 5(*H*)2-chloro-9,10-methlenedihydroxyphenanthridin-6-one.

205. The method of claim 184, wherein said compound is 5(*H*)2-nitro-9,10-methlenedihydroxyphenanthridin-6-one.

206. The method of claim 184, wherein said composition is in the form of a capsule or tablet containing a single or divided dose of said compound, wherein said dose is sufficient to prevent or reduce the effects of vascular stroke or other neurodegenerative disease.

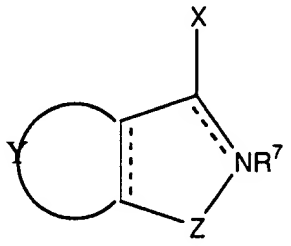
APPENDIX A
PENDING CLAIMS

207. The method of claim 184, wherein said composition is administered as a sterile solution, suspension or emulsion, in a single or divided dose.

208. The method of claim 184, wherein said composition is administered as a solid implant capable of releasing the compound over a prolonged period of time.

209. The method of claim 184, wherein said compound is present in an amount sufficient to treat or prevent neural tissue damage resulting from cerebral ischemia and reperfusion injury.

210. A method of effecting a neuronal activity in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:



I

and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

APPENDIX A
PENDING CLAIMS

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

211. The method of claim 210, wherein the neuronal activity is not mediated by NMDA.

212. The method of claim 210, wherein the neuronal activity is selected from the group consisting of stimulation of damaged neurons, promotion of neuronal regeneration, prevention of neurodegeneration, and treatment of a neurological disorder.

APPENDIX A
PENDING CLAIMS

213. The method of claim 212, wherein said neuronal activity is stimulation of damaged neurons resulting from cerebral ischemia or reperfusion injury.

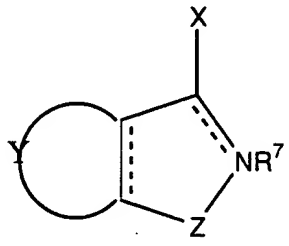
214. The method of claim 212, wherein the neurological disorder is selected from the group consisting of peripheral neuropathy caused by physical injury or disease state, traumatic brain injury, physical damage to the spinal cord, stroke associated with brain damage, demyelinating disease and neurological disorder relating to neurodegeneration.

215. The method of claim 214, wherein the neurological disorder relating to neurodegeneration is selected from the group consisting of Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis.

216. The method of claim 210 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

APPENDIX A
PENDING CLAIMS

217. A method of treating inflammation in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:



I

and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

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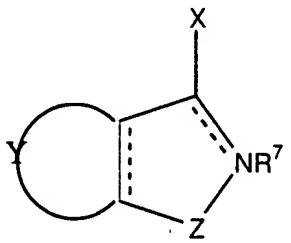
218. The method of claim 217, wherein said inflammation is colitis.

219. The method of claim 217, wherein said inflammation is Crohn's disease.

220. The method of claim 217 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

221. A method of treating a cardiovascular disorder in a mammal comprising administering to said mamal an effective amount of a compound of formula I containing at least one ring nitrogen:



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and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

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or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

222. The method of claim 221 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

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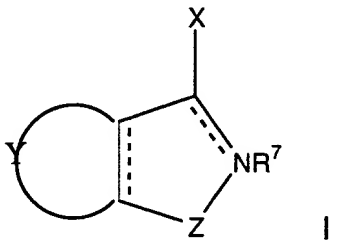
223. The method of claim 221, wherein said cardiovascular disorder is selected from the group consisting of coronary artery disease, angina pectoris, myocardial infarction, cardiogenic shock, and cardiovascular tissue damage.

224. A method of treating septic shock in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:



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and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

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Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

225. The method of claim 224 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

226. The method of claim 224, wherein said septic shock is endotoxic shock.

227. A method of treating diabetes in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:

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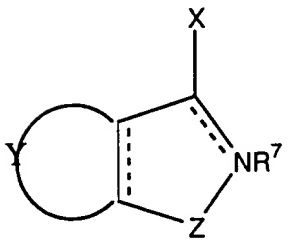

I

and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

228. The method of claim 227 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an

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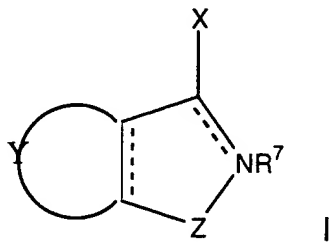
alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

229. A method of treating arthritis in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:



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and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

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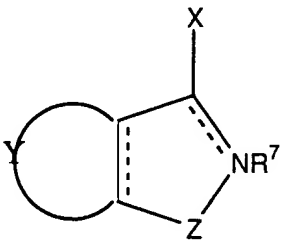
Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent.

230. The method of claim 229 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino, amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

231. A method of treating cancer in a mammal comprising administering to said mammal an effective amount of a compound of formula I containing at least one ring nitrogen:



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and having an IC_{50} of 100 μM or lower for inhibiting poly(ADP-ribose) polymerase *in vitro*,

or a pharmaceutically acceptable base or acid addition salt, prodrug, metabolite, optical isomer or stereoisomer thereof, wherein:

X is double-bonded oxygen or -OH;

R^7, when present, is hydrogen;

Y represents the atoms necessary to form a fused phenyl, pyridine, or pyrimidine ring; and

Z is $-R^6C=CR^3-$ wherein R^6 and R^3, taken together, form a fused phenyl, pyridine, or pyrimidine ring;

wherein said fused phenyl, pyridine, or pyrimidine ring of Y or Z is independently unsubstituted or substituted with at least one non-hydrogen, non-interfering substituent; and

when the Y and Z rings are phenyl and X is double bonded oxygen, at least one of the Y and Z rings contains at least one said substituent.

232. The method of claim 231 wherein said substituent is selected from the group consisting of an alkyl, an alkenyl, an alkynyl, a cycloalkyl, an aralkyl, an aryl, an alkoxy, an alkenoxy, an aryloxy, an aralkyloxy, an alkanoyl, a haloalkyl, a heterocyclic group, a heteroaryl, a halo group, hydroxy, carboxy, carbonyl, amino, an alkylamino,

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amido, cyano, isocyano, nitro, nitrilo, isonitrilo, nitroso, imino, azo, diazo, sulfonyl, sulfoxy, SO_3K, thio, thiocarbonyl, alkylthio and sulfhydryl.

233. The method of claim 231, wherein said cancer is selected from the group consisting of: ACTH-producing tumors, acute lymphocytic leukemia, acute nonlymphocytic leukemia, cancer of the adrenal cortex, bladder cancer, brain cancer, breast cancer, cervix cancer, chronic lymphocytic leukemia, chronic myelocytic leukemia, colorectal cancer, cutaneous T-cell lymphoma, endometrial cancer, esophageal cancer, Ewing's sarcoma, gallbladder cancer, hairy cell leukemia, head & neck cancer, Hodgkin's lymphoma, Kaposi's sarcoma, kidney cancer, liver cancer, lung cancer (small and/or non-small cell), malignant peritoneal effusion, malignant pleural effusion, melanoma, mesothelioma, multiple myeloma, neuroblastoma, non-Hodgkin's lymphoma, osteosarcoma, ovary cancer, ovary (germ cell) cancer, prostate cancer, pancreatic cancer, penis cancer, retinoblastoma, skin cancer, soft-tissue sarcoma, squamous cell carcinomas, stomach cancer, testicular cancer, thyroid cancer, trophoblastic neoplasms, cancer of the uterus, vaginal cancer, cancer of the vulva and Wilm's tumor.

APPENDIX B

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Ischemic Brain Injury Is Mediated by the Activation of Poly(ADP-Ribose)Polymerase

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Summary: Poly(ADP-ribose)polymerase (PARP, EC 2.4.2.30), an abundant nuclear protein activated by DNA nicks, mediates cell death *in vitro* by nicotinamide adenine dinucleotide (NAD) depletion after exposure to nitric oxide. The authors examined whether genetic deletion of PARP (PARP null mice) or its pharmacologic inhibition by 3-aminobenzamide (3-AB) attenuates tissue injury after transient cerebral ischemia. Twenty-two hours after reperfusion following 2 hours of filamentous middle cerebral artery occlusion, ischemic injury was decreased in $PARP^{-/-}$ and $PARP^{+/-}$ mice compared with $PARP^{+/+}$ litter mates, and also was attenuated in 129/SV wild-type mice after 3-AB treatment compared with controls. Infarct sparing was accompanied by functional recovery in $PARP^{-/-}$ and 3-AB–treated mice. Increased poly(ADP-ribose) immunostaining observed in ischemic cell nuclei 5 minutes after reperfusion was reduced by 3-AB treatment. Levels of NAD—the substrate of PARP—were reduced 2 hours after reperfusion and were 35% of contralateral levels at 24 hours. The decreases were attenuated in $PARP^{-/-}$ mice and in 3-AB–treated animals. Poly(ADP-ribose)polymerase cleavage by caspase-3 (CPP-32) has been proposed as an important step in apoptotic cell death. Markers of apoptosis, such as oligonucleosomal DNA damage, total DNA fragmentation, and the density of terminal deoxynucleotidyl transferase dUTP nick-end–labelled (TUNEL +) cells, however, did not differ in ischemic brain tissue of $PARP^{-/-}$ mice or in 3-AB–treated animals versus controls, although there were differences in the number of TUNEL-stained cells reflecting the decrease in infarct size. Thus, ischemic brain injury activates PARP and contributes to cell death most likely by NAD depletion and energy failure, although the authors have not excluded a role for PARP in apoptotic cell death at earlier or later stages in ischemic cell death. Inhibitors of PARP activation could provide a potential therapy in acute stroke. **Key Words:** Apoptosis—Cerebral ischemia—DNA damage—Energy depletion—Mice—Poly(ADP-ribose) polymerase.

Poly(ADP-ribose)polymerase (PARP, EC 2.4.2.30), an abundant nuclear protein present in all nucleated cells, is believed to be involved in DNA repair and participates in cell proliferation, differentiation, and transformation. Poly(ADP-ribose)polymerase catalyzes the covalent attachment of ADP-ribose subunits from its substrate, nicotinamide adenine dinucleotide (NAD), to numerous nuclear proteins, including PARP itself. Formation of poly(ADP-ribose) is a unique post-translational modification that can be induced by DNA strand breaks after exposure to nitric oxide (NO) or oxygen-free radicals (de Murcia et al., 1992, 1994; Lindahl et al., 1995; Shall, 1995).

The "PARP suicide hypothesis" states that excessive poly(ADP-ribose) formation due to PARP activation by DNA strand breaks and subsequent depletion of its substrate NAD might contribute to cell death through an energy failure mechanism. This may explain the neurotoxic properties of NO (Zhang et al., 1994) and why pancreatic islet cells die after exposure to reactive oxygen species (ROI) *in vitro* (Radons et al., 1994). In fact, inhibitors of PARP, such as 3-aminobenzamide (3-AB), can partially prevent NO and ROI cytotoxicity in neuronal and pancreatic cells. Nitric oxide and ROI formed during ischemia/reperfusion are known DNA-damaging agents and are potent mediators of cell death (Beckman et al., 1990).

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Abbreviations used: 3-AB, 3-aminobenzamide; CPP-32, cysteine protease putative protein; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; PARP, poly(ADP-ribose)polymerase; PBS, phosphate-buffered saline; ROI, reactive oxygen intermediates; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick-end labelling.

Recently, PARP was recognized as a substrate of activated caspase-3 (CPP-32), a mammalian homologue of the *C. elegans* ced-3 death gene, during apoptosis (Tewari et al., 1995; Nicholson et al., 1995). The role of PARP in apoptosis, however, remains unclear and somewhat controversial. On the one hand, cleaved PARP appears to facilitate apoptosis, possibly by interrupting DNA binding and repair at an earlier step than internucleosomal DNA fragmentation (Patel et al., 1996). Cleavage decreases PARP enzymatic activity and could facilitate DNA laddering by upregulating Ca^{2+}/Mg^{2+}-dependent endonuclease as a consequence of reduced poly(ADP-ribosyl)ation (Yoshihara et al., 1975, Nicholson et al., 1995). However, inhibition of poly(ADP-ribosyl)ation of histone H1 by 3-AB abolishes internucleosomal DNA fragmentation *in vitro* (Yoon et al., 1996). Treatment with enzymatic inhibitors of PARP reportedly blocked or augmented apoptosis, depending on the paradigm (Kaufmann et al., 1994).

In the present study, we analyzed the function of PARP in cerebral ischemia *in vivo* by studying the effects of transient focal cerebral ischemia in PARP null mice and after 3-AB treatment, a widely used, nonselective PARP inhibitor. Poly(ADP-ribose)polymerase null mice are phenotypically normal and develop without obvious behavioral defects. Although DNA repair is efficient after ultraviolet or alkylating agents, primary fibroblasts proliferate more slowly. Moreover, the mice are susceptible to epidermal hyperplasia (Wang et al., 1995). Heller et al. (1995) demonstrated that 3-AB prevents ROI and NO cytotoxicity in $PARP^{+/+}$ but not in $PARP^{-/-}$ islet cells *in vitro*, identifying PARP as the target of 3-AB-'s cytoprotective effects.

We show in the present study that PARP deletion or inhibition of PARP enzyme activity promotes resistance to ischemic brain injury after middle cerebral artery occlusion (MCAO). We further measured poly(ADP-ribose) formation and NAD levels in ischemic tissue as well as markers of apoptosis in ischemic tissue to decipher the mechanism by which activation of PARP mediates ischemic brain injury.

METHODS

Physiology

Regional cerebral blood flow was measured by laser Doppler flowmetry (PF2B, Perimed, Stockholm, Sweden), along with arterial blood pressure and heart rate, as described (Hara et al., 1996, 1997a,b). Arterial blood samples (50 μL) were analyzed for pH, arterial oxygen pressure (PaO_2) and partial pressure of carbon dioxide ($PaCO_2$) using a blood gas/pH analyzer (Corning 178, Ciba-Corning Diagnostics, Medford, MA, U.S.A.). Core temperature was maintained at approximately 37°C with a thermostat (FHC, Brunswick, ME, U.S.A.) and a heating lamp (Skytron, Daiichi Shomei, Tokyo, Japan) until 1 hour after reperfusion and during the treatment and monitoring period.

Ischemia Model

Adult male 129/SV mice (18–20 g, Taconic farm, Germantown, NY, U.S.A.) were anesthetized for induction with 1.5% halothane and maintained in 1.0% halothane in 70% nitrous oxide (N_2O) and 30% oxygen (O_2) using a Fluotec 3 vaporizer (Colonial Medical, Amherst, NH, U.S.A.). $PARP^{-/-}$ mice and $PARP^{+/-}$ and $^{+/+}$ litter mates (generated and genotyped by Dr. Wang at the Institute of Molecular Pathology, Vienna, as described in Wang et al., 1995) were anesthetized with 0.1 mL chloral hydrate (7% w/w in phosphate-buffered saline [PBS] given intraperitoneally). Ischemia was induced by occlusion with a 8-0 nylon monofilament coated with resin/hardener mixture (Xantopren and Elastomer Activator, Bayer Dental, Osaka, Japan) as described previously (Hara et al., 1996). For filament withdrawal after 2 hours of ischemia, the animals were reanesthetized briefly with halothane. Twenty-two hours after reperfusion, animals were killed and the brains were frozen immediately in 2-methylbutane on dry ice for cryostat sections or directly divided into five coronal 2-mm sections using a mouse brain matrix (RBM-200C, Activational Systems, Ann Arbor, MI, U.S.A.). Infarction volume was quantitated with an image analysis system (M4, St. Catharines, Ontario, Canada) on 2% 2,3,5 triphenyltetrazolium chloride-stained 2-mm slices (3-AB experiments) or hematoxylin & eosin-stained cryostat sections (20 μm; $PARP^{-/-,+/-,+/+}$ experiments) and calculated by summing the volumes of each section determined directly (Huang et al., 1994) or indirectly using the following formula: contralateral hemisphere (mm^3) – undamaged ipsilateral hemisphere (mm^3) (Swanson et al., 1990). 3-Aminobenzamide (Sigma Chemical Co., St. Louis, MO, U.S.A.; IC_{50} on PARP activity = 33 μmol/L, 97% PARP inhibition at 5mmol/L) was dissolved in PBS (Poly Scientific, Bay Shore, NY, U.S.A.) at a concentration of 27.2 mg/mL (0.2 mol/L). Two μL of this solution (0.4 μmol) were injected intracerebroventricularly (bregma –0.9 mm lateral, –0.1 mm posterior, –3.1 mm deep) 10 minutes before ischemia using a Hamilton injection syringe (Fisher Scientific, Pittsburgh, PA, U.S.A.).

Neurological Deficits

Mice were tested for Neurological deficits and scored as described by Bederson et al. (1986) with the following minor modifications (Hara et al., 1996): 0 = no observable Neurological deficit (normal); 1 = failure to extent right forepaw (mild); 2 = circling to the contralateral side (moderate); and 3 = loss of walking or righting reflex (severe). Animals were rated by the operator and by a second rater blinded to the treatment protocol and group identity. Assessments were made after 30 minutes and after 24 hours.

Poly(ADP-ribose) Immunohistochemistry

Rabbit polyclonal antisera directed against purified poly(ADP-ribose) was provided by Dr. Kunihiro Ueda (Kyoto University). The antibody is specific for poly(ADP-ribose), and immunoreactivity reflects both the number of ADP ribosylation sites and poly(ADP-ribose) chain length (Ikai et al., 1980). Mice were anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally) and transcardially perfused with 4% paraformaldehyde. After immediate removal, brains were stored in the same buffer containing 20% sucrose, and free-floating sections (40 μm) were prepared on a freezing microtome. Immunohistochemistry was performed according to a three-stage avidin-biotin method with biotinylated goat antirabbit immunoglobulin G as secondary antibody and 3'-3'-diaminobenzidine as chromogene. The 3'-3'-diaminobenzidine reaction was intensified with 0.04% nickel chloride. The poly(ADP-ribose) polyclonal antibody was used at a concentration of 1:100.

Negative controls were performed by omission of either the primary or secondary antiserum.

Nicotinamide Adenine Dinucleotide Determination

For *in vitro* measurements, animals were killed and the brains were removed immediately and cut into five coronal 2-mm slices. Tissue was obtained from within the ischemic zone in the third and fourth coronal section (4–8 mm) and snap-frozen. The samples were weighed, and the tissue was disrupted by freezing and thawing followed by homogenization in 4 × volumes PBS. The homogenate was centrifuged for 20 minutes at 15,000 × g at 4°C, and the supernatant was stored at −70°C until further use (Heller et al., 1995). Cellular NAD was determined by an enzymatic cycling method using alcohol dehydrogenase (Boehringer Mannheim, Mannheim, Germany) from *Saccharomyces cerevisiae*, as described previously (Nisslbaum and Green, 1969). Levels in ischemic tissue are expressed as percent of contralateral side. Contralateral values were stable at 0.00744 ± 0.00135 units/minute per milligram of tissue and did not differ between groups ($PARP^{+/+}$, $PARP^{-/-}$, 3-AB, PBS). For validation, NAD was added to homogenate prepared from ischemic and control tissue. Measurements increased linearly by addition of external NAD to the homogenate.

For qualitative *in situ* analysis, we adapted the method for tissue sections. The reaction mixture needed for enzymatic cycling was added to a 2% low-melt agarose solution at 45°C. Alcohol dehydrogenase was used at a final concentration of 50 units/mL, all other components were used at the same final concentration as in the *in vitro* assay. Glass slides were covered with 200 μL of the warm mixture in a homogeneous layer (approximately 1 mm thick) and kept horizontally on ice. Within 30 minutes, 12-μm cryostat brain sections were cut and placed onto the agarose-covered slide, immediately incubated at 37°C for 15 to 30 minutes in a humid chamber, and analyzed with the M4 image analysis system.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labelling (TUNEL)

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling was performed according to the method of Gavrieli et al. (1992) with minor modifications according to Wood et al. (1993). Terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP were obtained from Boehringer Mannheim (Mannheim, Germany). The biotinylated dUTP was visualized by the avidin-biotin method with 3′-3′-diaminobenzidine as chromogene. For negative controls, either TdT or biotinylated dUTP was omitted. For positive controls, the sections were treated with DNase I.

DNA Analysis

For DNA analysis, samples were prepared from the ischemic area and contralateral hemisphere at 24 hours. The brain was cut with a brain matrix, and the second (2–4 mm) slice was placed on a chilled glass plate. Tissue preparation was performed under a dissecting microscope, and only tissue from within the pale ischemic area was isolated for DNA preparation; corresponding tissue was taken from the contralateral side.

For quantitation of DNA damage, a terminal deoxynucleotidyl transferase-dependent isotopic end-labelling method was used (Tilly and Hsueh, 1993) with minor modifications as described previously (Hara et al., 1997b). Three micrograms of DNA were used in the labelling procedure together with 35 ng of a 100-base pair DNA fragment as the internal standard. The DNA was electrophoresed on a 2.0% agarose gel (agarose 3:1, Amresco, Solon, OH, U.S.A.), autoradiographed together with a radioactive standard, and analyzed with the M4 image analysis system. DNA less than 10 kilobase was used as an index of total DNA fragmentation. To measure oligonucleosomal damage more specifically, laddered DNA <1000 base was measured by summing the areas of each peak (areas under the curve) minus baseline densitometry readings. The method of quantitation was validated using an artificial "smear-ladder" system in which smeared DNA was obtained by extracting DNA from a decapitated mouse brain incubated at 37°C for 48 hours (MacManus et al., 1996). In a total amount of 1 μg of DNA, 0.02, 0.04, 0.08, and 0.12 μg of a commercially available 200-bp ladder (Invitrogen, Carlsbad, CA, U.S.A.) was added to a constant amount of smeared DNA. In another experiment, increasing amounts of smeared DNA (0.3, 0.5, 0.7, 0.9 μg) were added to a constant amount of ladder (0.1 μg). The readings for "total DNA fragmentation" and "oligonucleosomal damage" were related linearly to the amount of total (smeared) and laddered DNA (error < 10%).

Statistical Analysis

Data are presented as mean ± standard deviation. Statistical comparisons were made by two-tailed Student's *t*-test (infarct size, DNA quantitation) or by two-way analysis of variance followed by Student's *t* test (NAD determination) or one-way analysis of variance followed by Tukey test (physiology) or Dunnett's test (regional cerebral blood flow). For Neurological deficits, the Mann–Whitney rank sum test was applied to compare two groups, and Kruskal–Wallis one-way analysis of variance on ranks followed by Dunnett's test was used for three groups. Analysis was made using the software EXCEL (Microsoft, Redmond, WA, U.S.A.) or SigmaStat (Jandel, Corporation, San Rafael, CA, U.S.A.). $P < 0.05$ was considered statistically significant.

RESULTS

Physiologic Data

No significant differences in mean arterial blood pressure, heart rate, RCBF, or arterial blood gases were detected between 3-AB– and vehicle-treated 129/SV mice (n = 5 per group; Table 1). Regional cerebral blood flow

TABLE 1. *rCBF, mean arterial blood pressure, and physiological parameters before, during, and after 2 hours of MCA occlusion and 3-aminobenzamide treatment*

	PBS	3-AB	
rCBF (during)	18.3 ± 4.8	16.4 ± 3.0	[%]
rCBF (after)	96.3 ± 27.9	102.1 ± 16.2	[%]
MABP (before)	102.6 ± 6.0	96.4 ± 8.6	[mm Hg]
MABP (during)	102.2 ± 6.0	96.2 ± 11.3	[mm Hg]
MABP (after)	95.2 ± 8.4	92.2 ± 9.0	[mm Hg]
pH (before)	7.27 ± 0.03	7.25 ± 0.03	
pH (end)	7.32 ± 0.03	7.31 ± 0.05	
$Paco_2$ (before)	48.8 ± 7.0	47.9 ± 6.5	[mm Hg]
$Paco_2$ (end)	44.3 ± 5.8	46.9 ± 3.4	[mm Hg]
$Paco_2$ (before)	185.1 ± 19.9	195.1 ± 12.9	[mm Hg]
$Paco_2$ (end)	138.2 ± 25.4	124.6 ± 33.2	[mm Hg]

Mean arterial blood pressure (MABP) was measured before, during, and until 60 minutes after ischemia. Fifty microliter blood samples were withdrawn twice, before ischemia and just before reperfusion. Data are represented as mean ± SD. There are no significant differences between groups. MCA, middle cerebral artery; rCBF, regional cerebral blood flow.

decreased to less than 20% of baseline immediately after MCAO and sustained during 2 hours of ischemia. After reperfusion, RCBF increased to 90% to 100% of baseline within 5 minutes.

Ischemic Infarction in Poly(ADP-Ribose)Polymerase Null Mice

Twenty-two hours after reperfusion, direct infarct volume was significantly smaller in PARP null mice (n = 8) and PARP heterozygotes (n = 8) compared with wild-type litter mates (n = 8; Fig. 1A). Infarction area was significantly smaller in all five coronal sections in PARP null mice and in section 5 in PARP heterozygote mice compared with wild-type litter mates (Fig. 1B). Statistical significance also was achieved with an indirect method to determine infarction (71.7 ± 20.4 mm^3 [$PARP^{-/-}$], 76.5 ± 20.1 mm^3 [$PARP^{+/-}$], 110.3 ± 7.4 mm^3 [$PARP^{+/+}$], $P < 0.01$). All animals exhibited a Neurological score of 2 or higher 30 minutes after the onset of ischemia and after animals recovered from anesthesia. At 24 hours, the deficit was significantly smaller in PARP null mice compared with wild-type mice (0.7 ± 0.9 vs. 1.6 ± 0.8, $P < 0.01$). The deficit was less but did not reach statistical significance in the PARP heterozygote mice (1.0 ± 0.8 vs. 1.6 ± 0.8).

Infarction Volume in 3-Aminobenzamide–Treated Animals

3-aminobenzamide pretreatment (n = 8) significantly reduced direct infarct volume and area (sections 1–4) in 129/SV mice compared with PBS-injected control mice (n = 7) 22 hours after reperfusion (Figs. 1C and 1D). Also, infarct volume determined indirectly was significantly smaller (50.6 ± 12.0 mm^3 vs. 94.3 ± 19.2 mm^3, $P < 0.01$). Neurological deficits were significantly im-

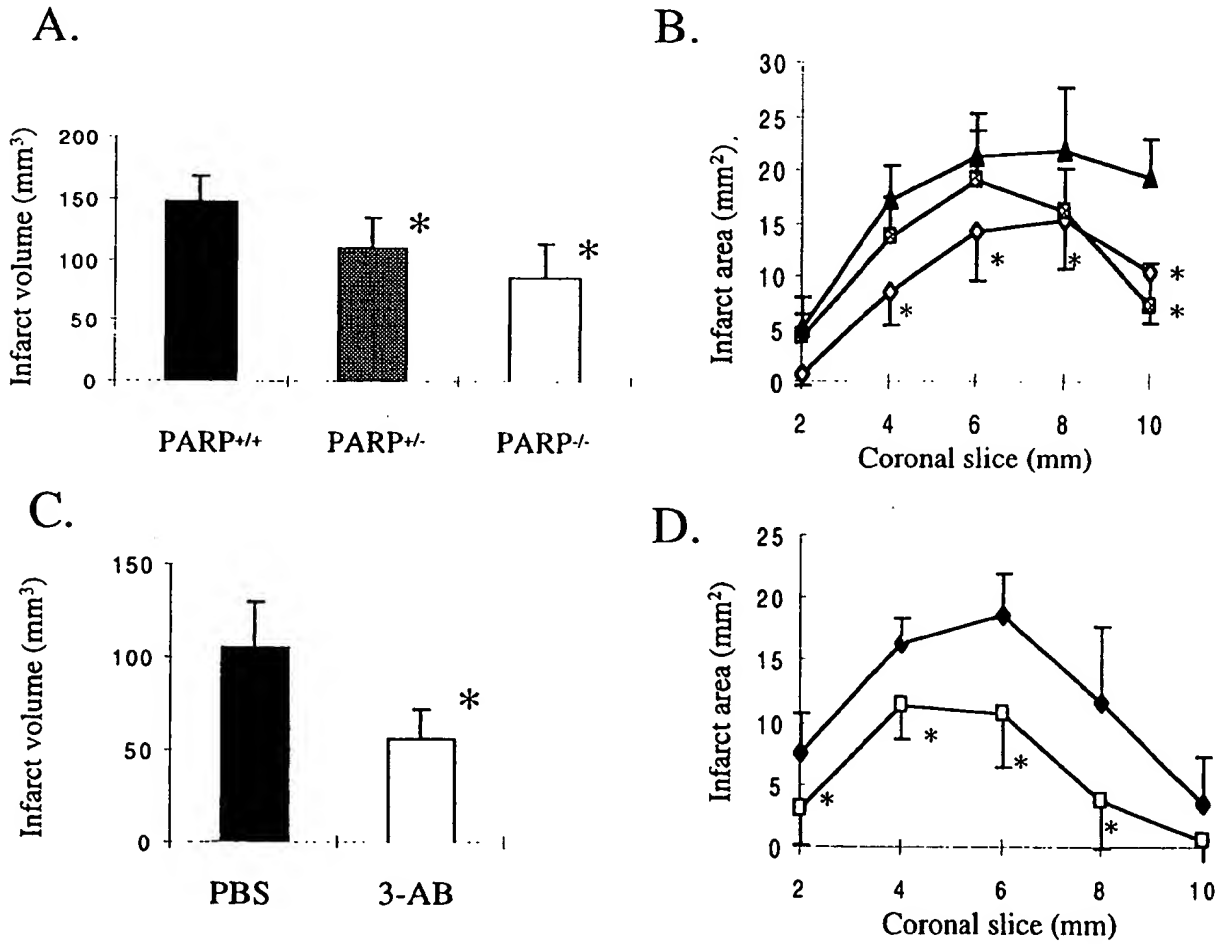

FIG. 1. Infarct size was smaller in Poly(ADP-ribose)polymerase $(PARP)^{-/-}$ and 3-aminobenzamide (3-AB)-treated animals compared with their respective controls 22 hours after 2-hour middle cerebral artery occlusion. Brain infarct volume (A) and brain infarct area (2-mm coronal sections) (B) were decreased in $PARP^{-/-}$ (diamonds) and $PARP^{+/-}$ (squares) compared with $PARP^{+/+}$ mice (triangles). Brain infarct volume (C) and infarct areas (D) were reduced after 3-AB (squares) compared with vehicle treatment (diamonds). 3-aminobenzamide (0.4 μmol) was injected intracerebroventricularly 10 minutes before ischemia. Data are presented as mean ± standard deviation (n = 7–8/group). * $P < 0.05$ vs. vehicle.

proved in 3-AB–treated animals (1.3 ± 0.5 vs. 2.3 ± 0.5, $P < 0.05$).

Poly(ADP-Ribose) Formation

To analyze PARP activation after ischemia, poly-(ADP-ribose) immunohistochemistry was performed. The number of poly(ADP-ribose) positive cells that could be identified clearly by specific nuclear staining increased two- to threefold in the ischemic tissue compared with the contralateral side 5 minutes after reperfusion following 2-hour ischemia. Also, the intensity of staining per cell was increased. Positive cells frequently showed swelling and nuclear disruption as early signs of cell damage. Poly(ADP-ribose) formation after MCAO and reperfusion was inhibited strongly by 3-AB (Fig. 2); staining was not detected in PARP null mice. After longer periods of reperfusion (3 or 6 hours) after 2-hour ischemia, or after 1-hour ischemia without reperfusion, increased poly(ADP-ribose) formation was not observed (not shown).

Nicotinamide Adenine Dinucleotide Determination

Brain levels of NAD did not differ under basal conditions between PARP null mice and wild-type litter mates and 3-AB versus vehicle-treated controls as assessed both *in vitro* and *in vivo*. Levels of NAD decreased to approximately 35% within the ischemic territory at 24 hours in vehicle-treated and $PARP^{+/+}$ mice. Higher NAD levels were measured in ischemic territory of PARP null mice compared with wild-type ($n = 5$ in duplicate per group), and in 3-AB–treated mice compared with vehicle controls ($n = 4$ in duplicate per group) after 24 hours (Fig. 3B and D).

Decreased NAD staining was observed in the middle cerebral artery territory as early as 2 hours after recirculation and was further decreased at 4 and 6 hours (Fig. 4). The decrease was attenuated after 3-AB treatment (Fig. 4). The decreased NAD staining preceded gross morphologic damage on hematoxylin and eosin stained sections (not shown).

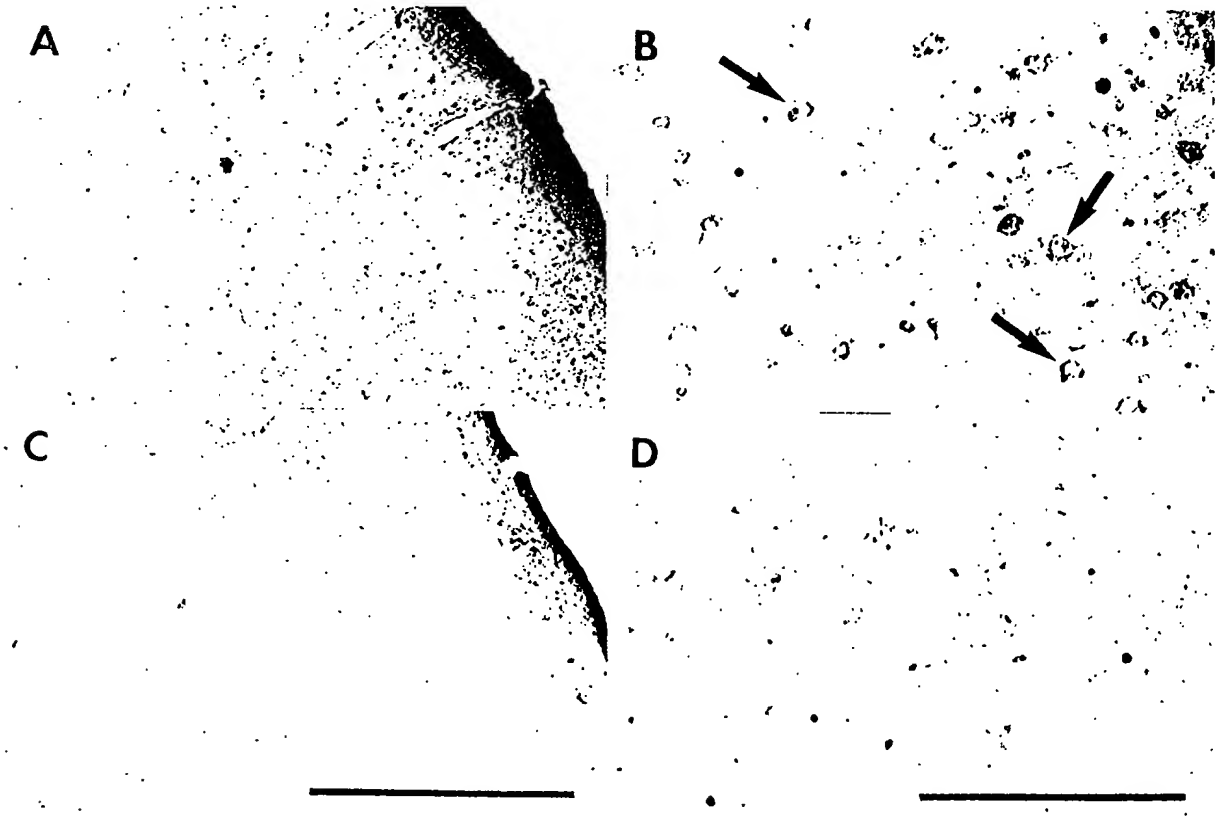

FIG. 2. Poly(ADP-ribose) immunoreactivity was increased in ischemic tissue 5 minutes after reperfusion following 2-hour middle cerebral artery occlusion. Poly(ADP-ribose) was visualized using a polyclonal poly(ADP-ribose) antibody. **(A)** Low and **(B)** higher magnification of ischemic tissue (vehicle-group); **(C)** low and **(D)** higher magnification of ischemic tissue after 3-aminobenzamide (3-AB) treatment (see Fig. 1). Cells show nuclear staining, prominent in the margins of the nucleus (indicated by arrows, B). The number of poly(ADP-ribose)-positive cells and the intensity of staining per cell was increased in ischemic tissue **(A, B)** compared with the contralateral side (not shown) Pretreatment with 3-AB **(C, D)** inhibited formation of poly(ADP-ribose) compared with the vehicle group **(A, B)**. Staining was not detected in $PARP^{-/-}$ mice (not shown, see also Wang et al., 1995). Scale bar = 1 mm **(A, C)**, 250 μm **(B, D)**.

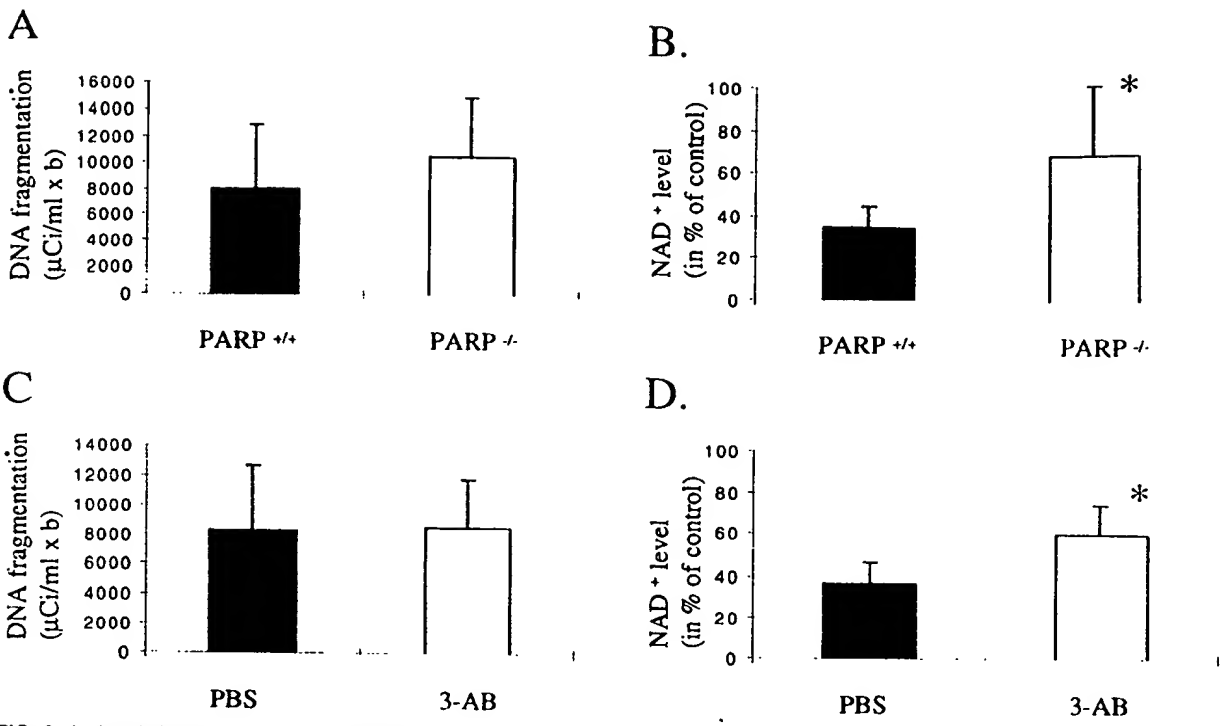

FIG. 3. Ischemic brain tissue from poly(ADP-ribose)polymerase (PARP)$^{-/-}$ mice and 3-aminobenzamide (3-AB)-treated mice exhibited the same amount of DNA fragmentation **(A, C)** but had significantly higher nicotinamide adenine dinucleotide (NAD) levels **(B, D)** 22 hours after 2-hour middle cerebral artery occlusion compared with their respective controls. DNA fragmentation was measured using a radioactive end-labelling method by terminal transferase (Tilly and Hsueh, 1993). Units are μCi/mL × b (see also Methods). No significant differences were detected between PARP null mice and 3-AB–treated mice vs. their respective controls. Nicotinamide adenine dinucleotide was measured with an enzymatic cycling method according to Nisslbaum et al. (1969). Values are given as percent of control (contralateral hemisphere). Significant differences were seen between PARP null mice and 3-AB–treated animals and their respective controls. * $P < 0.05$.

DNA Damage and Oligonucleosomal Fragmentation (Laddering)

TUNEL(+) cells were present in great numbers throughout the ischemic tissue in all animals at 24 hours, especially in the peri-infarct zone (n = 3 for PARP$^{-/-}$ and $^{+/+}$, 3-AB– and vehicle-treated mice). Many of these cells showed typical features of apoptosis. The lesion areas containing TUNEL(+) cells were smaller in PARP$^{-/-}$ mice and 3-AB–treated animals compared with their controls, matching smaller infarction areas as determined by hematoxylin & eosin staining on adjacent sections (data not shown). Estimates of cell density within the ischemic area were made in animals from all four groups at the level of the anterior commissure and 2 mm posterior to the anterior commissure. No difference in cell density within the ischemic area was found between groups when evaluated by two observers blinded to the treatment groups.

Brain tissue from within the ischemic zone from PARP null mice, 3-AB–treated animals, and controls exhibited DNA laddering on agarose gels (Fig. 5). Total DNA fragmentation did not differ between PARP null mice (n = 5) and wild-type litter mates (n = 5) after 24 hours. Moreover, 3-AB–treated mice (n = 6) did not differ from vehicle-treated controls (n = 5) after 24 hours (Figs. 4A and 4C). The amount of laddered DNA (oligonucleosomal damage) did not differ between PARP$^{-/-}$ and $^{+/+}$ at 24 hours (20 ± 6 vs. 22 ± 16 μCi/mL × b, n = 5) and 3-AB–treated mice compared with controls at 24 hours (30 ± 11 vs. 24 ± 14 μCi/mL × b, n = 6 or 5).

DNA fragmentation and DNA laddering also did not differ in 3-AB–treated animals compared with controls at 12 and 72 hours (n = 3 each). DNA fragmentation at 12 hours was 4250 ± 1770 versus 4110 ± 1310 μCi/mL × b and DNA laddering was 13 ± 3 versus 16 ± 12 μCi/mL × b (3-AB vs. PBS, respectively). DNA fragmentation at 72 hours was 23,700 ± 6450 versus 20,740 ± 9210 μCi/mL × b, and DNA laddering was 61 ± 26 versus 53 ± 27 μCi/mL × b (3-AB vs. PBS).

DISCUSSION

The present study provides both pharmacologic and genetic evidence that poly(ADP ribosyl)ation contributes to ischemic cell death, and that PARP inhibition protects tissue within the ischemic territory. Mice deficient in PARP were resistant to brain injury after transient focal

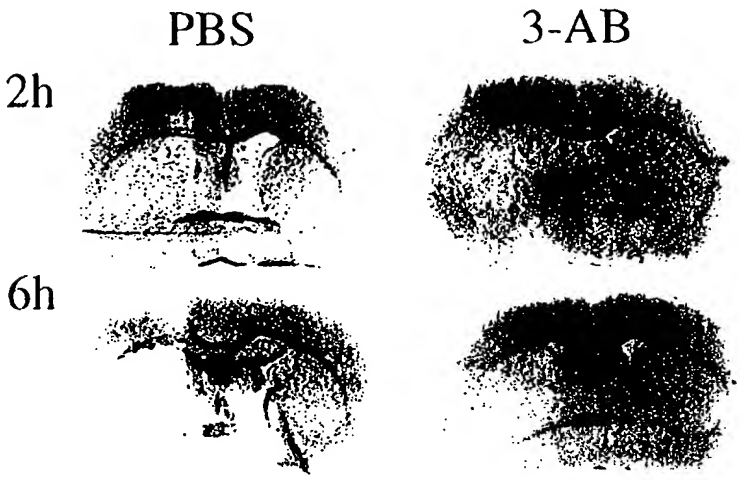

FIG. 4. The decreases in nicotinamide adenine dinucleotide (NAD) staining after middle cerebral artery occlusion (MCAO) were attenuated after 3-aminobenzamide (3-AB) pretreatment. Nicotinamide adenine dinucleotide histochemistry was performed in coronal brain slices from phosphate-buffered saline-treated (left) and 3-AB–treated animals (right) 2 and 6 hours after 2-hour MCAO. Histochemical staining for NAD was performed with an enzymatic cycling method (Nisslbaum et al., 1969) modified for cryostat sections (12 μm). Nicotinamide adenine dinucleotide staining (dark color) is detected throughout the brain but is depleted in the territory of the left middle cerebral artery at 2 hours and more pronounced at 6 hours in vehicle-treated animals (left). Nicotinamide adenine dinucleotide depletion was less severe in 3-AB–treated animals (right).

cerebral ischemia. Pharmacologic inhibition by 3-AB also conferred resistance to injury. Infarct sparing in both experiments was accompanied by improved Neurological scores, suggesting that reduced PARP activity preserves Neurological function. The differences between 3-AB and vehicle treated groups could not be explained by obvious effects on blood pressure or blood gases. These results recently have been confirmed by independent studies on $PARP^{-/-}$ mice (Eliasson et al., 1997, in press).

Poly(ADP-ribose) immunoreactivity increased in ischemic tissue but only at early time points after reperfusion (5 minutes). The increase in immunoreactivity in vehicle-treated animals did not sustain over time, possibly because of its short half-life (de Murcia and Ménisser-de Murcia, 1994). As expected, $PARP^{-/-}$ mice are deficient in poly(ADP-ribose) and remain so after MCAO. 3-aminobenzamide–treated mice also showed decreased poly(ADP-ribose) formation, which is in agreement with Heller et al. (1995). Increases were less dramatic without reperfusion, as was protection by 3-AB treatment in a model of permanent ischemia (not shown). Thus, the extent of neuroprotection after PARP inhibition/deletion may depend on the injury paradigm and the production of ROI and NO on reperfusion.

Poly(ADP-ribose)polymerase activation and subsequent depletion of NAD seem to be sequential events after the onset of reperfusion. Nicotinamide adenine dinucleotide levels decreased shortly after reperfusion in wild-type animals. Both 3-AB–treated mice and PARP

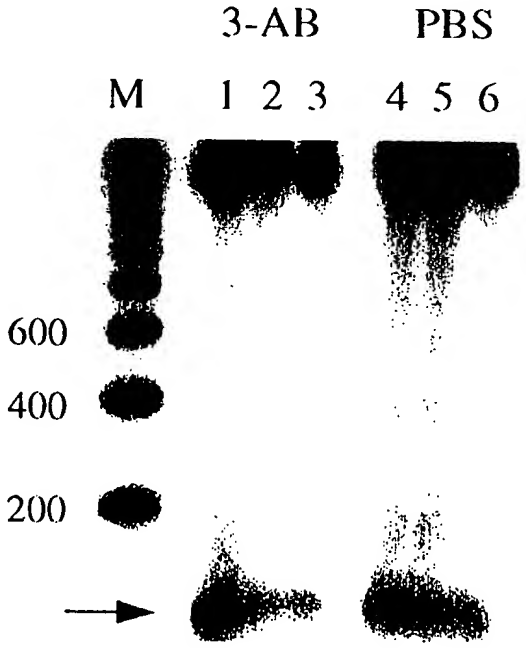

FIG. 5. Ischemic brain tissue from 3-aminobenzamide (3-AB)- and phosphate-buffered saline (PBS)-treated animals exhibited DNA laddering on agarose gel autoradiography 22 hours after middle cerebral artery occlusion. DNA was isolated from ischemic tissue and a [^{32}P]ddATP-end-labelling method with terminal transferase was performed. M: 200-bp standard ladder; Lane 1, 2, 3: individual 3-AB–treated animals; lane 4, 5, 6: individual PBS-treated animals; arrow indicates 100 bp internal standard. Quantitative analysis showed no significant difference between groups.

knockout mice exhibited higher levels within the middle cerebral artery territory, suggesting that the mechanism of protection may relate to energy sparing. Our measurements did not reflect the redox state of the tissue because we measured total NAD and not NADH/NAD^+. Resynthesis of NAD contributes to energy depletion because four molecules of adenosine triphosphate are needed to generate one molecule of NAD (Zhang et al., 1994). Under global ischemic conditions (Siesjö et al., 1973; Pulsinelli and Duffy, 1983; Nowak et al., 1985), levels of adenosine triphosphate and adenosine, and indices of mitochondrial function decrease minutes after global ischemia and return toward normal levels after 10 to 20 minutes, but not after longer ischemic periods (Phillis et al., 1996).

Current data suggest that oxidative and deaminative DNA damage develops early during reperfusion, and that this is distinct from DNA laddering/oligonucleosomal damage. DNA damage is mediated in part by NO, ROI, and possibly other metabolites, causing base modifications, and single- and double-strand breaks (Tamir et al., 1996). Liu and colleagues (1996) provided evidence for free radical-mediated DNA damage during transient forebrain ischemia, e.g., 8-hydroxy-2′-deoxyguanosine formation and DNA strand breaks increase during reperfusion. We observed random DNA fragmentation early after reperfusion whereas DNA laddering indicative of apoptosis was first evident 6 hours after 2 hours of focal cerebral ischemia and increased thereafter (Fink et al., 1997). Because DNA damage as judged by total DNA fragmentation, did not differ between groups in the present study, this might indicate that DNA repair does not occur early after ischemia or that DNA repair mechanisms are not impaired in PARP knockout and 3-AB–treated animals, which is consistent with previous results (Wang et al., 1995).

Recently, several laboratories provided experimental evidence for apoptotic cell death in the ischemic borderzone after MCAO (Li et al., 1995a,b; Charriaut-Marlangue et al., 1996; MacManus et al., 1996). Moreover, inhibition of the CED-3 mammalian homologues, caspase-3 (CPP-32) and caspase-1 (interleukin-1β–converting enzyme) attenuates infarction after transient focal cerebral ischemia (Hara et al., 1997a), and smaller infarcts also were observed in transgenic mice expressing a dominant negative mutant of interleukin-1β–converting enzyme (Friedlander et al., 1997; Hara et al., 1997b). Interestingly, PARP is a substrate for caspase-3, exhibiting only residual activity after cleavage. However, the functional role of PARP cleavage remains unclear. Because we did not observe differences in TUNEL cell density or DNA laddering within ischemic tissue in $PARP^{-/-}$ and $^{+/+}$ mice or 3-AB–treated animals compared with controls, our results suggest that if PARP deletion/inhibition blocks apoptosis in ischemia, it is not as robust as after administering peptide inhibitors of the interleukin-1β–converting enzyme family (Endres et al., 1997). The decrease in the number of TUNEL-stained cells in the knockout/treatment group probably only reflects infarct sparing because the density of positive cells did not differ between groups. These findings are consistent with studies by Liu et al. (1997), demonstrating that DNA fragmentation does not require the cleavage of PARP, and the fact that PARP knockout animals show no detectable defect in apoptosis during development (Wang et al., 1995). Of course, caspase-3 cleaves numerous substrates, one or more of which might mediate apoptosis in ischemia by events unrelated to PARP cleavage.

Thus, PARP activation contributes to ischemic cell death in brain, and the mechanism—although still not completely understood—may relate primarily to the consequences of depleting NAD levels. When given early, PARP inhibitors may be promising drugs for the treatment of acute stroke in humans.

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Dihydroisoquinolinones: the design and synthesis of a new series of potent inhibitors of poly(ADP-ribose) polymerase*

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Summary: A series of dihydroisoquinolinones, formally rigid analogs of 3-substituted benzamides, and a series of 2,3-disubstituted benzamides were synthesized and evaluated as inhibitors of poly(ADP-ribose) polymerase. The results indicated that the orientation of the amide with respect to the substituent on the aromatic ring was critical for optimum inhibitory activity. Selected compounds were also evaluated for their ability to modify the radiation response of mammalian cells to ionizing radiation. A number of the 5-substituted dihydroisoquinolinones, which were very potent inhibitors of the enzyme, were able to enhance the lethal effects of ionizing radiation in mammalian cells, as measured by changes in the survival curve parameters D_o and/or D_q.

The resistance of certain populations of tumor cells to the lethal effects of DNA damaging agents such as ionizing radiation has been attributed to efficient cellular repair of the induced damage (Harris, 1985). Inhibition of such repair should therefore have the potential for increasing the therapeutic benefit achievable by chemotherapy or radiotherapy. A number of enzymes have been implicated for their involvement in DNA repair or its regulation. Of these, one enzyme that has received considerable attention is poly(ADP-ribose) polymerase (ADPRP). This chromatin-bound enzyme catalyses the synthesis of poly(ADP-ribose) from NAD^+ (Hayaishi & Ueda, 1977). While the exact biological functions of ADPRP are not clearly understood, the activity of this enzyme has been shown to be elevated in the presence of DNA damage, suggesting that it may regulate, or directly participate in, the repair of DNA damage (Benjamin & Gill, 1980). The use of specific inhibitors of ADPRP has provided further evidence supporting a role for this enzyme in the repair of DNA damage (Shall, 1988). Known inhibitors of the enzyme, such as 3-aminobenzamide (3-AB) **6**, have been shown to potentiate radiation-induced lethality in mammalian cells (Ben-Hur *et al.*, 1984; Thraves *et al.*, 1985), to potentiate the cytotoxicity of certain chemotherapeutic agents (Chen & Pan, 1988; Kato *et al.*, 1988), and to inhibit the repair of DNA strand breaks (James & Lehman, 1982; Zwelling *et al.*, 1982; Ahnstrom & Ljungman, 1988). Ben-Hur *et al.* (1985) have also shown that the ability of a number of benzamide analogs to enhance radiation-induced cell killing is directly correlated with their potency as inhibitors of ADPRP. Therefore, this enzyme provides a

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Needed for good inhibitory activity

Preferred when R' = CH_3, Et, propyl

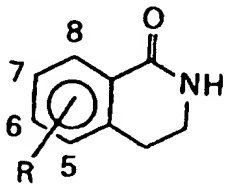
Figure 2

Materials and methods

Chemistry

The dihydroisoquinolinones were synthesized as indicated in Tables I and II. Compound **1a** was synthesized from **2a** (Nakagawa *et al.*, 1977) using sodium hydroxide and dimethyl sulfate. Compound **2d** was synthesized starting with phenol using a procedure similar to that described by Krollpfeiffer & Schultze (1924) and von Auwers & Hillinger (1916) (Scheme 1). The 5-nitrodihydroisoquinolinone **3a** was obtained by treatment of

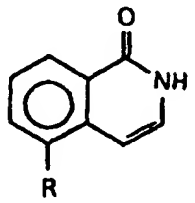
Table I Enzyme inhibition data for the dihydroisoquinolinones



Compound number	*R*	*Method of preparation*	IC_{50} (μM)
1a	5-OMe	A	0.42
b	7-OMe	B	120
c	6-OMe	B	39
2a	5-OH	C	0.10
b	7-OH	D	9.5
c	6-OH	D	2.0
d	8-OH	A	11
3a	5-NO_2	A	3.2
b	7-NO_2	D	13
4a	5-NH_2	A	0.41
b	7-NH_2	E	8.0
5	H	F	1.5
6	3-Aminobenzamide		9.0

A, see experimental section, B, Sall *et al.* (1987), C, Nakagawa *et al.* (1977), D, Tomita & Minami (1969), E, Eberlein *et al.* (1978), F, Griehl & Hecht (1958)

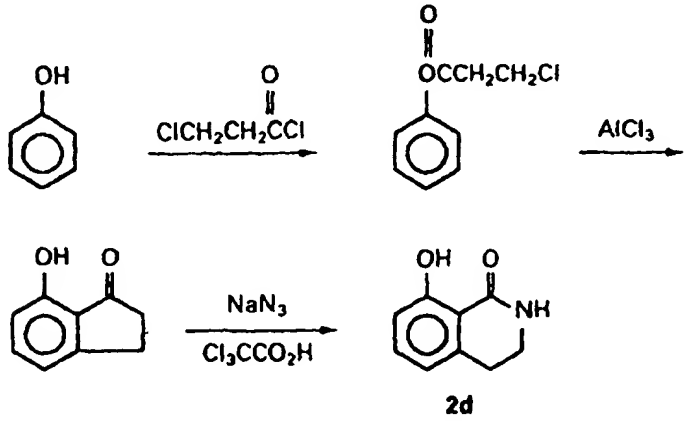
Table II Enzyme inhibition data for the isoquinolinones



Compound number	*R*	*Method of preparation*	IC_{50} (μM)
7	OH	A	0.14
8	OCH_3	B	0.58
9	NO_2	C	3.2
10	NH_2	C	0.24
11	H	A	6.2

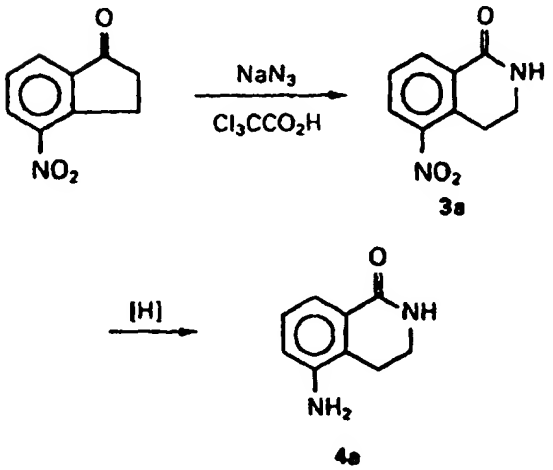
A, Aldrich Chemical Co.; B, Robinson (1947); C, Wenkert *et al.* (1964)

Scheme 1



2d

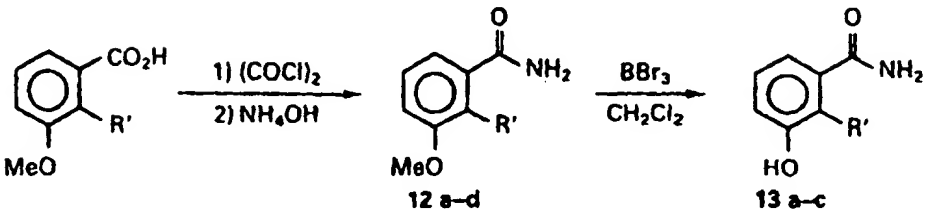
Scheme 2



3a

4a

Scheme 3



4-nitroindanone (Hasbun *et al.*, 1973) with sodium azide in trichloroacetic acid. Catalytic reduction using palladium on carbon gave **4a** (Scheme 2).

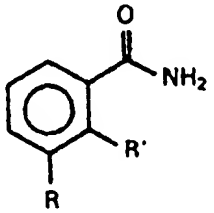
The synthesis of the 2,3-disubstituted benzamides is illustrated in Scheme 3. Treatment of the carboxylic acid derivatives, synthesized as previously described by Meyers *et al.* (1978), with oxalyl chloride, followed by ammonium hydroxide provided **12a–d**. Demethylation with boron tribromide cleanly gave the desired hydroxy benzamides **13a–c** (Table III). The 3-hydroxy-2-vinyl analog could not be obtained due to polymerization of the compound under the required reaction conditions.

Experimental

Chemical synthesis

All melting points were obtained on a Thomas–Hoover capillary melting point apparatus and are uncorrected. ^{1}H n.m.r. spectra were recorded on a Varian Associates EM-390,

Table III Enzyme inhibition data for the 2,3-disubstituted benzamides



Compound number	*R*	*R'*	*Method of preparation*	IC_{50} (μM)
12a	OMe	CH_3	A	N
b	OMe	Et	B	N
c	OMe	propyl	A	N
d	OMe	vinyl	A	N
3a	OH	CH_3	A	590
b	OH	Et	A	N
c	OH	propyl	A	N
14	OH	H	—	2.0

A, See Experimental Section; B, Herman (1944); N = not active (IC_{50} > 1 mM)

XL-200 or an IBM WP 100sy spectrometer using $CDCl_3$ or DMSO-d_6 as the internal reference standard. Purity was determined by microanalysis and by t.l.c. (silica gel 60F 254, Merck). Silica gel chromatography utilized Kieselgel 60 (70–230 mesh or 230–240 for flash chromatography). I.r. spectra were recorded with a Nicolet FT-IR spectrophotometer and mass spectra were determined on a VG analytical 7070E/HF or Finnegan 4500 mass spectrometer. All compounds possessed analytical data consistent with the proposed structures.

3,4-Dihydro-5-methoxy-1(2H)-isoquinolinone (1a). To a refluxing solution of 3,4-dihydro-5-hydroxy-1(2H)-isoquinolinone **2a** (Nakagawa *et al.*, 1977) (5.5 g, 33.7 mmol) in 2 N sodium hydroxide (35 ml) and methanol (70 ml) was added dimethyl sulfate (4 ml, 42.2 mmol). Additional sodium hydroxide (20 ml) and dimethyl sulfate (1 ml) were added after 2 h (three times). The mixture was then concentrated, diluted with water and acidified with sulfuric acid. The white solid which formed was collected and dried to provide **1a** (5.3 g, 89%). The sample was recrystallized from acetone; m.p. 146–149°C; n.m.r. ($CDCl_3$) δ 2.97 (t, 2H), 3.54 (m, 2H), 3.87 (s, 3H), 6.73 (bs, 1H), 7.04 (d, 1H), 7.32 (t, 1H), 7.72 (d, 1H), Anal. ($C_{10}H_{11}NO_2$) C,H,N.

3,4-Dihydro-8-hydroxy-1(2H)-isoquinolinone (2d). A mixture of phenol (50 g, 0.53 mol) and 3-chloropropionyl chloride (66 g, 0.53 mol) was heated at 80° for 16 h. The mixture was then allowed to cool and saturated sodium bicarbonate (500 ml) was added. The mixture was stirred for 1 h at 25°C, diluted with water (500 ml) and then extracted with ether. The ether layer was washed with brine, dried ($MgSO_4$), filtered and concentrated to provide the crude ester (90.9 g, 93%).

The ester was mixed with $AlCl_3$ (200 gm 1.50 mol) and heated at 150° for 10 h. The resulting black mass was cooled and carefully treated with dilute HCl and steam distilled to provide a solid. The solid was recrystallized from methanol to give 7-hydroxyindanone (3.3 g, 5%); m.p. 108–110°C with acceptable analytical and spectral data.

A mixture of 7-hydroxyindanone (3.11 g, 23.6 mmol) and trichloroacetic acid (39 g) was heated at 60–65°C until the mixture became homogeneous. Then sodium azide (2.3 g, 35.4 mmol) was added in one portion and heating was continued for 18 h. The mixture was cooled, diluted with ice/water and extracted with ether. The ether layer was dried ($MgSO_4$), filtered and concentrated to give 2.4 g of a dark oil. Flash chromatography (CH_2Cl_2) provided **2d** (0.87 g, 23%); m.p. 147–149°C; n.m.r. ($CDCl_3$) δ 2.88 (t, 2H), 3.40 (m 2H), 6.72 (d, 2H), 7.34 (t, 1H), 8.43 (bs, 1H), 12.79 (s, 1H); MS m/e = 163; Anal. ($C_9H_9NO_2 \cdot 0.05\ H_2O$) C,H,N.

3,4-Dihydro-5-nitro-1(2H)-isoquinolinone (3a). 4-Nitroindanone (3.0 g, 26.9 mmol, Hasbun, 1973) was added to trichloroacetic acid (90 g) which was preheated to 90°C. The mixture was stirred for 30 min and then sodium azide (2.8 g, 42.3 mmol) was added and heating continued for 4 h. The mixture was cooled and diluted with ice/water. The resulting solid was filtered, washed with water, dried and recrystallized from ethanol to give **3a** (1.34 g, 41%); m.p. 196–199°C; n.m.r. ($CDCl_3$) δ 3.12 (t, 2H), 3.38 (m, 2H), 7.60 (t, 1H), 8.17 (dd, 1H), 8.22 (dd, 1H), 8.28 (bs, 1H); MS m/e = 192; Anal. ($C_9H_8N_2O_3$) C,H,N.

5-Amino-3,4-dihydro-1(2H)-isoquinolinone (**4a**). A mixture of 3,4-dihydro-5-nitro-1(2H)-isoquinolinone **3a** (1.0 g, 5.2 mmol), ethanol (100 ml), and 5% palladium on carbon (0.2 g) was hydrogenated for 5 h, under 3 atmospheres of hydrogen. The mixture was filtered, concentrated and the residue was recrystallized from ethanol/hexane to provide ***4a*** (0.50 g, 70%). An analytical sample was prepared by dissolving the compound in ethanol, treatment with a solution of ethanolic HCl and filtering the resulting solid; m.p. 284–392°C;

n.m.r. (DMSO-d_6) δ 2.97 (t, 2H), 3.40 (m, 2H), 7.43 (t, 1H), 7.48 (dd, 1H), 7.60 (dd, 1H), 8.10 (bs, 1H); MS m/e = 162; Anal. ($C_9H_{10}N_2O$·HCl) C,H,N,Cl.

General procedure for the preparation of benzamides (12a–d) from the corresponding carboxylic acids. Synthesis of 3-methoxy-2-methylbenzamide (12a). To a solution of 3-methoxy-2-methylbenzoic acid (4.0 g, 24.1 mmol, Meyers *et al.*, 1978) in toluene (100 ml) was added oxalyl chloride (3.7 g, 29.1 mmol) in toluene (20 ml) dropwise. The mixture was stirred at 25°C for 1 h and then concentrated. The resulting solid was dissolved in THF (25 ml) and added dropwise to ammonium hydroxide (75 ml) at 0°C. The mixture was stirred for 3 h, diluted with water and the solid was filtered (if no solid formed the product was obtained by extraction with ether, drying the organic layer and concentrating to a solid). The filtrate was concentrated to a solid and was combined with that collected above to provide 3.6 g of material. Recrystallization from water gave **12a** (2.8 g, 41%); m.p. 159–162°C; n.m.r. (DMSO-d_6) δ 2.19 (s, 3H), 3.79 (s, 3H), 6.89 (d, 1H), 6.93 (t, 1H), 7.16 (d, 1H), 7.37 (bs, 1H), 7.66 (bs, 1H); MS m/e = 165; Anal. ($C_9H_{11}NO_2$) C,H,N.

2-Ethyl-3-methoxybenzamide (12b). Prepared as described above in a yield of 84%; m.p. 115–117°C (toluene/hexane) (Richtzenhain, 1944, m.p. 114°C); n.m.r. (DMSO-d_6) δ 1.10 (t, 3H), 2.65 (q, 2H), 3.80 (S, 3H), 6.87 (dd, 1H), 7.00 (dd, 1H), 7.18 (t, 1H), 7.32 (bs, 1H), 7.67 (bs, 1H); MS m/e = 179; Anal. ($C_{10}H_{13}NO_2$) C,H,N.

3-Methoxy-2-propylbenzamide (12c). Prepared as described above in a yield of 94%; m.p. 110–113°C (methanol/water); n.m.r. (DMSO-d_6) δ 0.87 (t, 3H), 1.50 (m, 2H), 2.63 (m, 2H), 3.79 (s, 3H), 6.87 (d, 1H), 7.00 (d, 1H), 7.19 (t, 1H), 7.32 (s, 1H), 7.67 (s, 1H); MS m/e = 193; Anal. ($C_{11}H_{15}NO_2$) C,H,N.

2-Ethyl-3-hydroxybenzamide (13b). Prepared as described above in a yield of 73%; m.p. 139–150°C; n.m.r. (DMSO-d_6) δ 0.87 (t, 3H), 2.47 (q, 2H), 6.54 (dd, 1H), 6.62–6.67 (m, 2H), 7.11 (bs, 1H), 7.44 (bs, 1H), 9.20 (bs, 1H), MS m/e = 165; Anal. ($C_9H_{11}NO_2$·0.2 H_2O) C,H,N.

General procedure for the preparation of 3-hydroxy-2-substituted benzamides from the corresponding 3-methoxy analogs. Preparation of 3-hydroxy-2-methylbenzamide (13a). To a solution of 3-methoxy-2-methylbenzamide **12a** (2.07 g, 12.5 mmol) in methylene chloride (50 ml) at 0°C was added boron tribromide (9.45 g, 37.6 mmol, 1 M BBr_3 in methylene chloride) dropwise. The mixture was allowed to warm slowly to room temperature and then stirred for 18 h. Methanol was carefully added and the mixture was concentrated. The resulting solid was recrystallized from water to provide **13a** (1.42 g, 75%); m.p. 165–171°C; n.m.r. (DMSO-d_6) δ 2.14 (t, 3H), 6.74–6.84 (m, 2H), 7.01 (t, 1H), 7.29 (s, 1H), 7.61 (s, 1H), 9.45 (s, 1H); MS m/e = 151; Anal. ($C_8H_9NO_2$) C,H,N.

2-Ethyl-3-hydroxybenzamide (13b). Prepared as described above in a yield of 73%; m.p. 139–150°C; n.m.r. (DMSO-d_6) δ 0.87 (t, 3H), 2.47 (q, 2H), 6.54 (dd, 1H), 6.62–6.67 (m, 2H), 7.11 (bs, 1H), 7.44 (bs, 1H), 9.20 (bs, 1H), MS m/e = 165; Anal. ($C_9H_{11}NO_2$·0.2 H_2O) C,H,N.

3-Hydroxy-2-propylbenzamide (13c). Prepared as described above in a yield of 64% (product was isolated by chromatography over silica gel using 5% methanol/methylene chloride); m.p. 136–138°C; n.m.r. (DMSO-d_6) δ 0.86 (t, 3H), 1.30–1.71 (m, 2H), 2.42 (m, 2H), 6.73 (dd, 1H), 6.85 (d, 1H), 7.00 (t, 1H), 7.24 (bs, 1H), 9.37 (s, 1H); MS m/e = 179; Anal. ($C_{10}H_{13}NO_2$) C,H,N.

Biological methods

Determination of IC_{50} values (ADPRP Inhibition). ADPRP was purified >878-fold from calf thymus using protamine sulfate precipitation followed by column chromatography on DNA agarose (Shizuta *et al.*, 1980). The specific activity was 25 units per mg/protein, where 1 unit of enzyme activity is defined as the amount polymerizing 1 nmol of ADP-ribose moiety from [^{3}H] NAD/min at 30°C. Enzyme assays for determination of IC_{50} values were carried out in 100 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 60 µg calf thymus DNA, 50 µg/ml histone, purified enzyme and varying amounts of inhibitor. Reactions were carried out at 30°C for 15 min upon the addition of 0.1 mM NAD containing 1.5 µCi/ml [Adenine-2,8-^{3}H]–NAD. Incorporation of label into TCA-precipitable material was then quantitated by liquid scintillation counting. The data are expressed as IC_{50} values, i.e. the concentration of compound required to inhibit 50% of the measured enzyme activity. All reported IC_{50} values represent the mean of at least two separate determinations in which the IC_{50} values varied less than two-fold between assays.

In vitro radiation survival experiments

Chinese hamster V79-171b cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum and 0.05 µg/ml gentamicin in a 5% CO_2/95% air humidified incubator. Cells were periodically checked and found free of *Mycoplasma* contamination. Stock cultures in exponential growth were detached by trypsinization and appropriate cell numbers were seeded in 60 mm Petri dishes and incubated approximately 18 h to permit cell attachment. Cell multiplicity was 1. Under the conditions described, the doubling time of these cells was approximately 10 h and the cloning efficiency ranged from 55–75%.

Cells were irradiated at room temperature with a Phillips X-ray source operated at 320 kV and 10 mA using a thoraeus filter (HVL = 2.0 mmCu). Dose rates were measured using a standard Victoreen electrometer and were approximately 1.5 Gy/min.

Cell survival was measured by standard clonogenic assay. Exponential cells were prepared as described above and were irradiated with graded doses of X-rays. Immediately following irradiation, appropriate concentrations of drug solutions were added directly to media in dishes. The dishes were returned to the incubator for 2 h followed by rinsing and media replacement for colony formation. After approximately 6–7 days, colonies were stained with a 0.5% solution of crystal violet in methanol. Colonies of 50 or more cells were scored as survivors. Surviving fraction was determined from colony counts of four dishes per data point in each experiment, corrected for the plating efficiency of unirradiated cells. Surviving fraction values represent data from a minimum of two experiments. Survival curves were constructed using the single hit, multitarget model, $S = 1 - (1 - e^{-D/D_0})^n$. The survival curve parameters D_0 and D_q were determined by fitting the model to the data using least squares linear regression analysis of survival data falling on the exponential portion of the curve. D_0 is a measure of the steepness of the terminal slope of the radiation survival curve, whereas D_q is taken as a measure of the width of the initial shoulder (non-exponential) region of the survival curve. A reduction in the value of either or both of these parameters represents an enhancement of radiation-induced cell killing.

Results

All of the compounds described were evaluated for their ability to inhibit ADPRP. Table I summarizes the data obtained for the first group of compounds, the dihydroisoquinoli-

nones. Substitution at the 5-position resulted in extremely potent inhibitors of the enzyme with IC_{50} values generally less than 0.5 μM; only the 5-nitro substituted compound proved to be an exception. The 6- and 7-substituted derivatives were significantly less potent, but still retained activity, with IC_{50} values similar to that of 3-aminobenzamide (~9 μM). The unsubstituted derivative (5-H) was also comparable in activity to 3-AB. The 5-substituted derivatives, **2a** and **4a**, were 50–75 fold more potent as inhibitors of the enzyme than the 7-substituted compounds, **2b** and **3b**. Table II summarizes the results obtained with a series of 5-substituted isoquinolinones. The effectiveness of these compounds at inhibiting the enzyme was comparable to that of the corresponding dihydro series. Only the unsubstituted isoquinolinone, **11**, was less potent (4-fold) than its dihydroisoquinolinone counterpart, **5**. Table III summarizes the data obtained for the 2,3-disubstituted benzamides. Only compound **13a** had any detectable inhibitory activity.

As discussed, previously known inhibitors of ADPRP such as 3-aminobenzamide (3-AB) have been shown to modify many biological processes including the response of cells to ionizing radiation. Since the dihydroisoquinolinones are significantly more potent inhibitors of the enzyme than 3-AB, a greater effect on radiation-induced cell lethality might be expected. Therefore, studies were designed and undertaken to examine the effects of a select group of dihydroisoquinolinones on the response of mammalian cells to ionizing radiation. As shown in Table IV, changes in the survival curve parameters D_o and D_q were used as a measure of the modification of this response. With respect to radiation curves, an increase in the slope (reduction in D_o) or a decrease in the width of the shoulder region (reduction in D_q) can be interpreted as an enhancement in radiation-induced lethality. The data reveal that the standard ADPRP inhibitor, 3-AB had a small but reproducible effect on cell survival, manifested as a reduction in the value of D_o only. The 5-substituted dihydroisoquinolinones **1a**, **2a**, and **4a** were shown to reduce significantly D_o and/or D_q. The 7-substituted dihydroisoquinolinones **2b**, **2c**, and **4b** had no effect on either radiation survival parameter.

Discussion

Since the nuclear enzyme, ADPRP, is thought to be involved in biochemical processes associated with DNA repair or its regulation, it provides an attractive target for

Table IV *In vitro* radiosensitization activity of the dihydroisoquinolinones

Compound number	*Concentration (mM)*	D_o	D_q
Control	—	1.90±0.05[a]	3.10±0.08
1a	3.0	1.64±0.11	2.57±0.13
1b	1.5	1.90±0.16	3.63±0.30
2a	1.5	1.82±0.10	2.29±0.12
2b	3.0	1.86±0.14	3.67±0.27
2c	0.75	1.88±0.10	3.28±0.18
4a	3.0	1.63±0.11	2.54±0.16
4b	3.0	2.05±0.15	3.40±0.25
7	3.0	1.46±0.10	3.41±0.24
6 (3-AB)	10	1.81±0.09	3.13±0.16

[a] Errors on the survival parameters represent the 95% confidence intervals

radiotherapy and chemotherapy. Previous work with ADPRP inhibitors such as 3-aminobenzamide (3-AB) has shown that inhibition of this enzyme results in diminished repair of DNA damage. However, the effect seen is often small and dependent upon the cell line and experimental protocol. The 5-substituted dihydroisoquinolinones described herein can be considered 'rigid' benzamide analogs, but they are significantly more potent inhibitors of the enzyme than compounds such as 3-aminobenzamide. By restricting the free rotation of the amide moiety via an ethane/ethene bridge, a 50–75 fold increase in inhibitory activity was observed. Restriction of the rotation alone, however, does not account for the increased potency. Positioning of the substituent on the aromatic ring is also critical for activity as was demonstrated by a loss of activity when the substituent was moved from the 5 to the 6, 7, or 8 position. Another possible explanation for the increased potency seen with the dihydroisoquinolinones could have been that the ethane bridge in these compounds merely provided bulk at the 2-position of the benzamide. However, as evidenced by a lack of activity of the 2,3-disubstituted benzamides, this is not the case. It is also important to note that while 2-substituents do restrict the rotation of the amide, preliminary energy calculations have shown that the substituents at the 2-position of 2,3-disubstituted benzamides cause the amide nitrogen to exist predominantly in an orientation unfavorable for potent activity (Figure 2B).

Our biological studies have shown that the 5-substituted dihydroisoquinolinones modify the response of mammalian cells to ionizing radiation *in vitro*. Currently, clinical radiotherapy regimens use daily low X-ray doses to minimize normal tissue damage and to allow tumor reoxygenation between doses. A major drawback to this approach lies in the fact that extensive repair can occur between X-ray doses. Therefore, the 5-substituted dihydroisoquinolinones, which are extremely potent at inhibiting the putative repair enzyme ADPRP, may prove clinically useful. Further support for the potential of the dihydroisoquinolinones as radiosensitizers comes from survival data indicating that some members of this series effectively reduce the size of the shoulder region of an X-ray survival curve (D_q; see Table IV). Since D_q is thought to be a measure of the inherent repair capacity of a given cell type, a reduction in D_q by the dihydroisoquinolinones is consistent with their role as DNA repair inhibitors.

The data obtained thus far suggest that the enhanced cell kill observed in the presence of the dihydroisoquinolinones is related to the potency with which they inhibit ADPRP. Relatively weak inhibitors such as 3-AB or the 7-substituted dihydroisoquinolinones have little or no effect on cell survival after exposure to radiation. Current work is focusing on the use of these new inhibitors to examine the role of ADPRP in the response of mammalian cells to DNA damage with the intent of exploring the potential therapeutic applications of these compounds as adjuncts to radiotherapy and certain forms of chemotherapy.

In summary, a new series of potent ADPRP inhibitors has been discovered. The data suggest that the increased potency of these compounds can be attributed to positioning of the amide moiety in an optimal orientation with respect to the substituent on the aromatic ring. Additional work is currently underway to further define the structural features necessary for potent inhibition of ADPRP and/or optimal radiosensitization.

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Specific Inhibitors of Poly(ADP-Ribose) Synthetase and Mono(ADP-Ribosyl)transferase*

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Two classes of enzymes, poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferases, catalyze covalent attachment of multiple or single residues, respectively, of the ADP-ribose moiety of NAD⁺ to various proteins. In order to find good inhibitors of poly(ADP-ribose) synthetase free of side actions and applicable to *in vivo* studies, we made a large scale survey using an *in vitro* assay system, and found many potent inhibitors. The four strongest were 4-amino-1,8-naphthalimide, 6(5*H*)- and 2-nitro-6(5*H*)-phenanthridinones, and 1,5-dihydroxyisoquinoline. Their 50% inhibitory concentrations, 0.18–0.39 μM, were about two orders of magnitude lower than that of 3-aminobenzamide that is currently most popularly used. A common structural feature among all potent inhibitors, including 1-hydroxyisoquinoline, chlorthenoxazin, 3-hydroxybenzamide, and 4-hydroxyquinazoline, in addition to the four mentioned above, was the presence of a carbonyl group built in a polyaromatic heterocyclic skeleton or a carbamoyl group attached to an aromatic ring. Most of the inhibitors exhibited mixed-type inhibition with respect to NAD⁺. Comparative studies of the effects on poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase from hen heterophils revealed high specificity of most of the potent inhibitors for poly(ADP-ribose) synthetase. On the other hand, unsaturated long-chain fatty acids inhibited both enzymes, and saturated long-chain fatty acids and vitamin K_1 acted selectively on mono(ADP-ribosyl)transferase. The finding of many inhibitors of ADP-ribosyltransferases, especially poly(ADP-ribose) synthetase, supports the view that ADP-ribosylation of proteins may be regulated by a variety of metabolites or structural constituents in the cell.

Poly(ADP-ribose) synthetase (also termed poly(ADP-ribose) polymerase or NAD⁺: protein(ADP-ribose)$_n$ ADP-ribosyltransferase) (EC 2.4.2.30) is a nuclear enzyme that catalyzes a transfer of the ADP-ribose moiety of NAD⁺ to acceptor protein with a concomitant release of nicotinamide (chain initiation), and then to this protein-bound ADP-ribose, forming a ribosyl(1″→2′)ribose bond (straight chain elongation) or, less frequently, a ribosyl(1″′→2″)ribose bond (chain branching) (1–5). Repetition of the elongation process results in poly(ADP-ribose) synthesis or poly(ADP-ribosyl)ation of protein. The enzymatic activity is unique in its absolute requirement for the presence of DNA with strand termini. Various nuclear proteins, including histones, nonhistone proteins, and the enzyme itself, serve as acceptors of poly(ADP-ribose) *in vitro* as well as *in vivo* (2–5). Poly(ADP-ribose) synthetase exists in the nucleus of almost all eucaryotic cells (2), ranging from protozoa to mammals (4, 5). Only terminally differentiated cells, such as mature granulocytes, epidermal cells, and intestinal epithelial cells, lack this enzymatic activity (6). Recently, cloning of cDNAs encoding poly(ADP-ribose) synthetases from several animal sources revealed the basic structure of the enzyme being conserved throughout evolution (7, 8). Biological roles suggested for poly(ADP-ribose) or poly(ADP-ribosyl)ation of proteins include implications in DNA repair (6, 9), cell differentiation (10–12), control of cell cycle (13, 14), transformation (15, 16), transcription (17, 18), and alteration of chromatin architecture (19–21). One effective way to investigate these or still unknown biological functions is to modulate the poly(ADP-ribose) synthetase activity *in vivo* with a specific inhibitor and analyze ensuing changes in cellular functions. Many compounds have been shown to inhibit the poly(ADP-ribose) synthetase activity *in vitro*, and several of them have been used *in vivo* (2, 4, 5, 22–27). Most of the known inhibitors, however, including 3-aminobenzamide, nicotinamide, and thymidine are accompanied by various *in vivo* side actions (2, 5, 28), and their use has left more or less inconclusiveness to the results. Therefore, researchers in this field are awaiting for more specific and potent inhibitors.

In this paper, we report *in vitro* effects of a large variety of compounds on the activity of poly(ADP-ribose) synthetase purified from bovine thymus. Furthermore, by comparing the effects on poly(ADP-ribose) synthetase and monomer/arginine-specific ADP-ribosyltransferase (mono(ADP-ribosyl)transferase) (EC 2.4.2.31) from hen heterophils (29), we show that many of the inhibitors newly found are highly specific for poly(ADP-ribose) synthetase, whereas some act selectively on mono(ADP-ribosyl)transferase or nonspecifically on both enzymes. A preliminary report of this study was published elsewhere (30).

EXPERIMENTAL PROCEDURES[1]

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[1] Portions of this paper (including "Experimental Procedures," part of "Results," Tables I–IV, Fig. 1, and Footnotes 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

has three functional domains, *i.e.* for DNA binding, automodification, and NAD^+ binding (7, 55). Our mixed-type inhibitors would act on both the enzyme and the enzyme-NAD^+ complex at site(s) distinct from the NAD^+-binding site.

Poly(ADP-ribose) synthetase reaction is limited to the initiation step at NAD^+ concentrations below 100 nM (47). We investigated actions of representative inhibitors from different chemical classes under this condition and found that all inhibitors inhibited also this step (*e.g.* Fig. 1, *A* and *B*). We failed to confirm marked activation of the initiation reaction reported for 3-aminobenzamide at very low concentrations (such as 12 or 30 nM) (47). Instead, we found potent stimulation by *p*-phthalic acid or harmaline at higher NAD^+ concentrations, particularly, in the absence of Mg^{2+} (Table II). A metal chelator, 1,10-phenanthroline (No. 76), was stimulatory to the enzyme activity in the presence of Mg^{2+}, but inhibitory in its absence (Table II). The latter effect may be related to the presence of two zinc atoms in poly(ADP-ribose) synthetase molecule (46).

In dealing with many compounds, we had one technical difficulty, that is, a low solubility in the aqueous medium. We used Me_2SO, and only this solvent, as a vehicle for compounds essentially insoluble in water because it could dissolve practically all compounds tested and was freely miscible with water. Me_2SO did not affect the mono(ADP-ribosyl)transferase activity but inhibited weakly the poly(ADP-ribose) synthetase activity by itself at the concentration of, or below, 10%, and diminished effects of other inhibitors to variable extents.[5] Therefore, a comparison of water-soluble and insoluble inhibitors was feasible by employing a minimal concentration (*e.g.* 2%) of Me_2SO.

Besides the use in studies of biological functions of poly(ADP-ribose), our new inhibitors of poly(ADP-ribose) synthetase may have a potential to be applied to chemotherapy of malignant diseases. There are two possible mechanisms by which the inhibitors might help cure malignancies, that is, induction of cell differentiation and interference with DNA repair. Our preliminary experiments have indicated that new inhibitors, such as retinal, arachidonic acid, and 4-hydroxyquinazoline, are capable of inducing differentiation of murine teratocarcinoma cells in culture, as are retinoic acid, 3-aminobenzamide, and nicotinamide (12). The molecular basis of this induction of cell differentiation by poly(ADP-ribose) synthetase inhibitors is not known at present but may be related to the phenomenon of a selective loss of oncogenes from transformed mouse NIH 3T3 cells after treatment with luminol (No. 13) (56) or human leukemic HL-60 cells with 4-hydroxyquinazoline (No. 10) (57). A role of poly(ADP-ribose) synthetase in DNA excision repair has been established by many lines of evidence (4, 6), including interference with ligation of damaged DNA (6) and potentiation of cytotoxic effects of γ-irradiation or alkylating agents by poly(ADP-ribose) synthetase inhibitors (6, 9). In fact, some of our new inhibitors proved to retard the cellular recovery from potentially lethal damage after γ-irradiation.[6]

Most of the very strong inhibitors acted on poly(ADP-ribose) synthetase rather than arginine-specific mono(ADP-ribosyl)transferase from hen heterophils. In contrast, vitamins K_1 and $K_{2(20)}$ and several saturated long-chain fatty acids inhibited more specifically mono(ADP-ribosyl)transferase than poly(ADP-ribose) synthetase. As judged by a marginal effect of sodium dodecyl sulfate, at 0.1 mM, on the mono(ADP-ribosyl)transferase activity (data not shown), fatty acids do not appear to inhibit the enzymatic activity merely by their detergent-like action. Furthermore, we observed that diacylglycerols (*e.g.* 1-stearoyl-2-arachidonoyl-*sn*-glycerol) and phospholipids (*e.g.* distearoyl-L-α-phosphatidyl-DL-glycerol) are potent inhibitors of mono(ADP-ribosyl)transferase ($IC_{50} < 5\ \mu M$). The former compounds are well known as activators of protein kinase C (58), indicating a possible link between this enzyme and mono(ADP-ribosyl)transferase in the cell. In this context, it is noteworthy that a very small change in the concentration of saturated long-chain fatty acid or diacylglycerol was enough to induce maximal inhibition of mono(ADP-ribosyl)transferase activity (data not shown). This finding may indicate that ADP-ribosylation system(s) respond sensitively to changes in the local concentrations of these membrane-derived signal compounds in the cell. Whether the cytotoxicity that we preliminarily found in murine teratocarcinoma cells with some of potent inhibitors (*e.g.* 1, 8-naphthalimide and vitamin K_5), but not others (*e.g.* 4-hydroxyquinazoline and arachidonic acid), is due to *in vivo* effects on ADP-ribosylation or else remains to be investigated.

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Inhibition Studies

Inhibitory effects on enzyme activities were examined under the standard conditions except for the addition of test compound at 0.1 or 1 mM. Control (usually 4000–4700 cpm incorporation) was the mean of duplicates with no test compound added. Effects of all compounds were examined on, at least, two independent experiments; the difference between the two experiments being < 5%. IC_{50} values were estimated for individual inhibitors graphically from reverse curves encompassing at least two points in the range of 30–70% inhibition and several controls. In addition, 50% inhibition was directly verified at the calculated IC_{50} concentration for some potent inhibitors. The compounds that were not completely water-soluble at specified concentrations were first dissolved in Me_2SO, and the solution was added to the reaction mixture. Final concentrations of Me_2SO (usually 2%, if added, and rarely 10%) are specified in Tables. Control for compounds dissolved in Me_2SO was taken by adding the same concentration of Me_2SO.

Analysis of Mode of Inhibition

Kinetics or modes of inhibition of poly(ADP-ribose) synthetase were studied at both macromolar and micromolar concentrations of NAD^+. The assay conditions for macromolar NAD^+ were the same as the standard ones except for varying concentrations of [*Ade*-U-^{14}C]NAD^+ (215,000 cpm), the use of enzyme from bovine thymus (1.0–1.5 μg), and incubations at 25°C for 30 s. The assay mixture with micromolar NAD^+ contained [*adenine*-^{32}P]NAD^+ (150,000 cpm) in place of [*Ade*-U-^{14}C]NAD^+. Kinetics data were analyzed by the least-squares regression method using a computer.

Correction for Color Quenching

Quenching of scintillation by colored compounds was corrected for by the external standardization method. First, trichloroacetic acid-insoluble material was collected on a glass fiber filter, and counted. Then, the filter was thoroughly washed with toluene, dried and, after application of a known quantity (35,000 cpm) of [*Ade*-U-^{14}C]NAD^+, counted a second time. Control was taken in duplicate.

RESULTS

Table I summarizes the effects of 132 compounds on the activity of poly(ADP-ribose) synthetase. Compounds were classified into three groups, *i.e.* miscellaneous, benzamides, and fatty acids and arranged, within each group, in order of IC_{50} values.

Most of inhibitors in the "miscellaneous" group belong to either of the following eleven chemical classes.

(1) Isoquinoline Derivatives and Analogues. Isoquinoline itself was a very weak inhibitor[3] (Table II), but some of its analogues or derivatives were the most potent so far found. The strongest was 4-amino-1,8-naphthalimide (No. 1),[4] followed by 6(5*H*)-phenanthridinone (No. 2), 2-nitro-6(5*H*)-phenanthridinone (No. 3), and 1,3-dihydroxyisoquinoline (No. 4). 1,8-Naphthalimide (No. 5) and 1-hydroxyisoquinoline (No. 7) were also very strong. *N*-Hydroxynaphthalimide (No. 41) was moderately strong, whereas *N*-(2-chloroethyl)-1,8-naphthalimide (No. 63) was weak. These results suggest that any substitution at the amide nitrogen of 1,8-naphthalimide decreases the inhibitory potency.

(2) Quinazoline Derivatives and Analogues. All tested derivatives of quinazolines (No. 6, 8, 10, or 16) except one (No. 67, 75) were very strong or strong inhibitors. A big difference in the inhibitory potency between benzoylanthranilic (No. 8) and 6,7-dimethoxyquinazoline-2,4-dione (No. 75) suggests that substituents at the 6- and 7-positions markedly decrease the potency. The parent compound, quinazoline[5] (3,4-benzodiazine) (No. 66), was weak. Cinnoline (1,2-benzodiazine) was more potent (IC_{50} = 1400 μM, data not shown), but this result needs re-examination because of colored impurity and susceptibility to air oxidation. Another mono-, quinoxaline (1,4-benzodiazine), was not inhibitory (data not shown).

(3) Coumarin Derivatives. Coumarin (No. 43) was a moderately strong inhibitor. The most potent in this group was chlorobenzoxazone (No. 9), a compound substituted at the 2 position by a chloromethylene group. Substitution at the 4- position by an acetamido group (No. 62) markedly decreased the inhibitory potency.

(4) Phthalazinone Derivatives and Analogues. Phthalazone[3] (1,2,3-benzodiazine) (No. 22), that is an isomer of quinazolines, was a moderately strong inhibitor. The most potent in this group was 4(2*H*)-phthalazinone (No. 11). Cinnoline (No. 13) (3*H*) was also very strong, and phthalhydrazide (No. 14) and 3-mercaptophthalhydrazide (No. 19) were strong. Phthalimide, that has a five-membered imide ring in place of a six-membered hydrazide ring (as in phthalhydrazide), did not exhibit the enzyme activity (data not shown). Indeed, oxidation of the hydrazide ring to the imide ring, suggesting a contribution of the amidic ring to the inhibitory action (data not shown).

(5) Chromone Derivatives and Analogues. Flavone (No. 12) and 2-methylchromone (No. 17) were very strong and strong inhibitors, respectively. Chromone-2-carboxylic acid (No. 46) and 4-chromanone (No. 52), a dihydro analogue of chromone, were moderately strong.

(6) Pyridazine Derivatives. 3-Iodocinnoline (No. 18) and 5-iodocinnoline (No. 20) were the strongest inhibitors in this group. 5-Bromocinnoline (No. 23), 5-bromocinnoline (No. 36), thymidine (No. 24), and thymine (No. 33) were moderately strong, as reported previously (10, 39). Two other compounds, 5-chloro- and 5-nitrouracils (No. 32, 40), were also moderately strong.

(7) Quinoline Derivatives. 4-Hydroxy-2-methylquinoline (No. 30) and 4-hydroxyquinoline (No. 21) were the strongest, followed by xanthurenic acid (No. 25) and kynurenic acid (No. 49). The latter two compounds are metabolites of tryptophan in mammals. The fact that all potent inhibitors have a hydroxyl group (or a keto group in an isosteric form) at the 4-position suggests the importance of an oxygen atom at this position for the inhibitory action.

(8) Nicotinamide Derivatives and Analogues. The strongest in this group were acetoxamide (No. 57), a well-known inhibitor (2, 5, 40), and its isomer, α-picolinamide (No. 30) (41). Another isomer, nicotinamide (No. 58) (42), was moderately strong, whereas an analogue, thionicotinamide (No. 64), was weak. We confirmed 3-methylnicotinamide (No. 37) as a moderately strong inhibitor (43), and 6-aminonicotinamide (No. 59) and 1-methylnicotinamide (No. 72) as weak inhibitors (24, 39). As exemplified by *N*-methylnicotinamide (24), alkylation of the carbamoyl (carboxamide) group drastically diminished the inhibitory potency of nicotinamide (data not shown).

(9) Naphthalene Derivatives and Analogues. 5-Hydroxy-1,4-naphthoquinone (No. 28) was the strongest in this group. All other tested derivatives of 1,4-naphthoquinone (No. 29) were also moderately strong. Naphthalene itself was not inhibitory at all (data not shown), suggesting the importance of oxo groups(s) on 1,4-naphthoquinone for inhibition. Substitutions at the 2- and/or 3-position decreased the inhibitory potency (No. 31, 36, 39, 45, 50, 51, 55).

In this group were included four members of vitamin K, *i.e.* K_3 (No. 59) (23), K_1 (No. 45), menadione sodium bisulfite (No. 31), and K_2 (No. 61). They were moderately strong or weak. We could purify vitamin K_2 only partially, and thus the data may be considered as tentative. Tetralone (No. 158), a 1-naphthalenone analogue with a cyclohexanone ring, and 1-indanone (No. 56), a benzocyclopentanone with a five-membered ring, were moderately strong. 1-Tetralone (No. 54), a saturated analogue, were moderately strong to weak.

(10) Coumarin Derivatives. Coumarin (No. 71) itself was a weak inhibitor. More potent were its 4-hydroxy or 4-amino derivatives (No. 47, 56). Novobiocin (No. 69) exhibited poly(ADP-ribose) synthetase activity at 1 mM (Table I) and strongly at 5 mM (Table II).

(11) Acetophenone Derivatives. Acetophenone (No. 69) was a weak inhibitor, as reported previously (22). The strongest among its derivatives was 4-hydroxyacetophenone (No. 48) (23), followed by 4-aminoacetophenone (No. 57) and 4-aminobenzophenone (No. 65) (23). It is noteworthy that the same trend of potency was seen among 3-substituted benzamides.

(12) Benzamide Derivatives and Analogues. Monosubstituted benzamides, particularly at the 3 position, were the most potent in this group. Among polysubstituted benzamides, only 3,4-dimethoxy- and 5-amino-salicylamides were strong (No. 86, 90). A comparison between 3-substituted benzamides and 3- or 5-substituted salicylamides indicates that an additional hydroxyl function decreases the inhibitory potency. As exemplified by 3,4-dimethoxy- and 3-methoxybenzamide (No. 92) and *N*,3-dimethylbenzamide (No. 126), methylation of the amide group and amino(benzamide) or the carbamoyl group of benzamide deactivated totally or partially the potency (see also Ref. 22). A phenylic substitution on the carbamoyl group of salicylamide abolished the inhibitory action, as shown by *N*-phenylsalicylamide (data not shown). Cyclohexanecarboxamide (No. 100) (21), a saturated analogue of benzamide, was moderately strong. Contrary to a report (22), we had no difficulty in solubilizing benzamide in water up to the concentrations used.

(13) Fatty Acids. Arachidonic acid (No. 121) and linoleic acid (No. 122) were the most potent inhibitors in this group (see also Ref. 44). Other unsaturated long-chain fatty acids with more than one double bond were also strong (No. 123, 124) or moderately strong (No. 125, 126). Linolenic acid (No. 127) and elaidic acid (No. 129) were somewhat less potent than their cis-isomers (No. 122, 123). The potencies of all saturated long-chain fatty acids so far tested (No. 128, 130, 131) were very weak.

Table II, see the next part.

Table III summarizes the effects of all compounds on the activity of nuclear ADP-ribosyltransferase purified from bovine testis. Compounds were classified into four groups, *i.e.* miscellaneous and fatty acids, and arranged according to the same rule as in Table I. In addition to 13 strongest inhibitors of poly(ADP-ribose) synthetase, we tested 6 general fatty acids, several vitamins, and some additional compounds. For descriptions of the results, see the next part.

Table IV shows a comparison between IC_{50} values of 28 selected compounds for poly(ADP-ribose) synthetase and nuclear ADP-ribosyltransferase. Compounds were arranged according to the rank of IC_{50} values for nuclear ADP-ribosyltransferase and poly(ADP-ribose) synthetase. For descriptions of the results, see the next part.

TABLE I

Effects of Various Compounds on Poly(ADP-Ribose) Synthetase Activity

Compound	IC_{50} (μM)	Inhibition (%) at 0.1 mM	Inhibition (%) at 1 mM
I. MISCELLANEOUS			
1 6-Amino-1,8-naphthalimide[a,b]	0.18	99	>99[c]
2 2*H*-Benz[*de*]isoquinolin-1-one (6(5*H*)-phenanthridinone)[a,b]	0.30	99	>99
3 2-Nitro-6(5*H*)-phenanthridinone[a,b]	0.35	92	>93
4 1,3-Dihydroxyisoquinoline[a]	0.39	99	100
5 2*H*-Benz[*de*]isoquinoline-1,3-dione (1,8-naphthalimide)[a,b]	1.4	98	98
6 2-Methyl-4(3*H*)-quinazolinone[a,b]	5.6	86	98
7 1-Hydroxyisoquinoline (isocarbostyril)[a]	7.0	82	98
8 2,4(1*H*,3*H*)-Quinazolinedione (benzoylanthranilic)[a,b]	8.1	81	98
9 Chlorobenzoxazole[a,b]	8.5	80	98
10 4-Hydroxyquinazoline[a]	9.5	80	95
11 4(3*H*)-Phthalazinone[a,b]	12	77	88
12 2-Phenylchromone (flavone)[a,b]	22	72	88
13 3-Aminophthalhydrazide (luminol)[a,b]	23	74	96
14 2,3-Dihydro-1,4-phthalazinedione (phthalhydrazide)[a,b]	30	70	95
15 5-Iodouracil[a]	43	66	89
16 2-Mercapto-4(3*H*)-quinazolinone[a,b]	44	89	94
17 2-Methyl-1,4-benzopyrone (2-methylchromone)[a]	45	60	83
18 5-Iodocinnoline[c]	71	35	84
19 3-Mercaptophthalhydrazide[a,b]	72	36	90
20 4-Hydroxy-2-methylquinoline[a,b]	74	35	87
21 4-Hydroxyquinoline[a,b]	80	39	86
22 2,3-Benzodiazine (phthalazine)[a]	130	44	78
23 5-Bromouracil[c]	160	42	74
24 Thymidine[a]	180	36	77
25 4,8-Dihydroxy-2-quinolinecarboxylic acid (xanthurenic acid)[a,b]	190	33	73
26 5-Bromocytidine[a]	210	5	73
27 Nicotinamide[c]	310	41	72
28 5-Hydroxy-1,4-naphthoquinone (juglone)[a,b]	350	12	>95
29 1,4-Naphthalic acid (1,4-naphthoquinone)[a,b]	350	24	87
30 α-Picolinamide[c]	350	-1	68
31 2,3-Dichloro-1,4-naphthoquinone[a,b]	360	36	>82
32 5-Chlorouracil[a]	370	33	71
33 5-Methyluracil (thymine)[a]	380	30	73
34 4-Aminophthalhydrazide (isoluminol)[c]	390	22	70
35 1,4-Dihydro-1(2*H*)-naphthalenone (α-tetralone)[a,b]	410	-1	67
36 2-Hydroxy-1,4-naphthoquinone (lawsone)[a,b]	430	26	74
37 3-Methylnicotinamide[a]	450	-1	64
38 1,4-Benzoquinone[c]	450	-3	78
39 2-Methyl-1,4-naphthoquinone (vitamin K_3, menadione)[a,b]	450	8	>70
40 5-Nitrouracil[a,b]	450	30	65
41 *N*-Hydroxynaphthalimide sodium salt[a]	450	8	>96
42 all-*trans*-Retinal (vitamin A aldehyde)[a,b]	450	10	>70
43 Carnosine (carbonylalkylamide)[a,b]	490	-1	66
44 4-Nitrophthalhydrazide[a,b]	510	-1	63
45 2-Methyl-3-phytyl-1,4-naphthoquinone (vitamin K_1, phytylmenadione)[a,b]	520	-1	>72
46 Chromone-2-carboxylic acid[a]	560	-1	62
47 4-Hydroxycoumarin[a,b]	570	17	62
48 4-Hydroxyacetophenone[c]	600	21	63
49 4-Hydroxy-2-quinolinecarboxylic acid (kynurenic acid)[a,b]	670	-1	61
50 5-Hydroxy-2-methyl-1,4-naphthoquinone (plumbagin)[a,b]	700	31	>82
51 Menadione sodium bisulfite[c]	720	-1	58
52 4-Chromanone[a,b]	720	-1	55
53 Resorcinol[a,b]	790	-1	>66
54 1-Tetralone[a,c]	810	-1	53
55 2-Amino-3-chloro-1,4-naphthoquinone[a,b]	830	-1	>93
56 4-Aminocoumarin[a,b]	850	-1	53
57 *m*-Aminoacetophenone[c]	930	30	65
58 Nicotinamide[a]	990	-1	51
59 6-Aminonicotinamide[c]	1100	-1	49
60 1,3-Dihydroxynaphthalene[c,d]	1300	-1	43
61 5-Hydroxy-2-methyl-1,4-naphthoquinone (vitamin K_2)[a,b]	1300	-1	>31
62 4-Acetamidocoumarin[a,b]	1400	-1	43
63 *N*-(2-Chloroethyl)-1,8-naphthalimide[a,b]	<1800[e]	-1	>37
64 Thionicotinamide[c]	1800	-1	43
65 *m*-Aminobenzophenone[c]	1900	-1	36
66 1,3-Benzodiazine (quinazoline)[a]	2000	-1	38
67 2-Trichloromethyl-4(3*H*)-quinazolinone[a,b]	2300	6	32
68 Novobiocin[c]	2300	-3	16
69 Acetophenone[c,d]	2300	-1	35
70 4-Hydroxypyridine[c]	2300	-1	34
71 1,2-Benzopyrone (coumarin)[a,b]	2800	-1	33
72 1-Methylnicotinamide chloride[c]	3800	-1	28
73 *trans*-Decahydro-1-naphthalenone (*trans*-1-decalone)[a,b]	4300	-1	22
74 Nicotinamide[c]	4700	-1	21
75 6,7-Dimethoxyquinazoline-2,4-dione[a,b]	--	-1	22
76 1,10-Phenanthroline[a,b]	--	-1	102

[3] In this paper, inhibitors are tentatively classified into five classes of potency, according to the IC_{50} values: IC_{50} < 25 μM, very strong; 25-100 μM, strong; 101-1000 μM, moderately stong; 1001-5000 μM, weak; and > 5000 μM, very weak.

[4] The number after "No." corresponds to the number given to the compound in Table I.

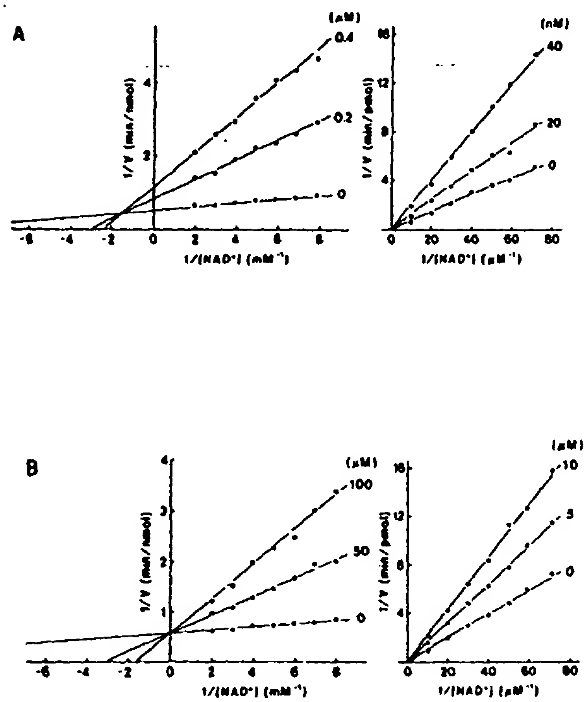

Fig. 1. Kinetics of poly(ADP-ribose) synthetase inhibition: (A) mixed-type inhibition by 6(5H)-phenanthridinone, and (B) competitive inhibition by xanthurenic acid. The final concentrations of Me_2SO in reactions with micromolar or nanomolar NAD^+ were 0.016 or [illegible] (A), and 0.5 or [illegible] (B) respectively.

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Effect of 6(5H)-phenanthridinone, an Inhibitor of Poly(ADP-ribose) Polymerase, on Cultured Tumor Cells

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Abstract. By catalyzing posttranslational modifications of nuclear proteins, poly(ADP-ribose) polymerase (PARP) controls their functions and therefore constitutes an enzyme of crucial importance in tumor development. In this study, we have investigated the action of 6(5H)-phenanthridinone, an isoquinoline derivative and one of the most potent PARP inhibitors described so far, on RDM4 murine lymphoma cells in culture. We also examined whether this compound could act synergistically with an antineoplastic drug in tumor-cell destruction. Our results demonstrate that a marked inhibition of PARP activity can be obtained in whole cells after a short incubation, and that this compound, when associated with an alkylating agent, dichloro-2,2′ *N*-methyldiethylamine (chloromethine), leads to a marked drop in the RDM4 proliferation, indicative of a synergy between the two compounds.

Key words: poly(ADP-ribose) polymerase, 6(5H)-phenanthridinone, nitrogen-mustard, lymphoma cells, proliferation.

Attempts to increase the efficiency of anticancer treatments by using substances directed against molecular targets distinct from, but close to, DNA constitute an old but still promising approach both to circumvent the resistance of tumor cells toward classical cytotoxic agents and to lower the active concentrations of the latter. Among these putative molecular targets is PARP[e] (EC 2. 4. 2. 30), a chromatin-bound enzyme, which plays a key role in a series of cellular functions involving DNA: cell proliferation [1, 2] and differentiation [3], DNA repair [4–6], cell death control [7, 8], and aging [9]. These effects result from the PARP-catalyzed covalent binding of ADP-ribose moieties to nuclear enzymatic or structural proteins, such as ligase, endonuclease, topoisomerase, or histones. In fact, it has been frequently reported that PARP inhibitors were able to enhance the efficiency of antitumor drugs by interfering with DNA repair mechanisms. So far, such attempts to reinforce the action of antiproliferative drugs have been principally achieved with "classical" PARP inhibitors, such as 3-MBA or nicotinamide. However, these inhibitors are only active at millimolar concentrations and are poorly specific, making them inappropriate for clinical use.

Recently, new types of PARP inhibitors have been demonstrated in cell-free systems. One of the most active compounds among them was 6(5H)-phenanthridinone, an isoquinoline derivative [10]. It blocks the activity of purified enzyme at micromolar concentrations.

In this paper, we show that 6(5H)-phenanthridinone is also able to inhibit PARP activity in RDM4 murine lymphoma cells within the same range of concentrations. Moreover, when combined with the alkylating agent chloromethine, it synergistically blocks the proliferation of these tumors cells.

MATERIALS AND METHODS

Tumor cell line. The cell line used in this study was RDM4, a murine lymphoma from AKR (spleen cell) origin. Cells of this tumor line were routinely grown in suspension in RPMI 1640 medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (DAP, Vogelgrün, France), gentamycin (50 μg/ml), sodium pyruvate (1 mM), and nonessential amino acids (Gibco). Doubling time was approximately 14 h. Cells were harvested during exponential growth.

Drugs and chemicals. 6(5H)-phenanthridinone was purchased from Aldrich Chimie S.a.r.l., Saint Quentin Fallavier, France. It was solubilized in dimethylsulfoxide and stored at room temperature as a 40 mM solution. 3-MBA was purchased from Sigma Chimie, Saint Quentin Fallavier, France. Dichloro-2,2′ *N*-methyldiethylamine (chloromethine) was obtained from Laboratoires Delagrange, Chilly Mazarin, France. [*Adenine*-2,8-^{3}H]NAD (33.9 mCi/mmol) and [^{3}H]-TdR (6.7 mCi/mmol) were purchased from Dupont-New England Nuclear, Hertfordshire, England.

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[e]*Abbreviations used:* PARP, poly(ADP-ribose) polymerase; ADP, adenosine diphosphate; 3-MBA, 3-methoxybenzamide; [^{3}H]-TdR, [^{3}H]-thymidine; EU, enzymatic unit; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; TNF, tumor necrosis factor.

PARP assay. Endogenous enzymatic activity of PARP was determined in cells permeabilized according to [11] by measuring the incorporation of the ADP-ribose moiety of [*adenine*-2,8-^{3}H]NAD$^+$ into acid-insoluble material as previously described [12]. The incubation mixture contained 120 mM Tris, pH 7.8, 4 mM $MgCl_2$, 10 mM DTT, 3 mM β-mercaptoethanol, 0.048% Triton-X100, 100 μM [*adenine*-2,8-^{3}H]NAD$^+$ (200 mCi/mmol), and 40 μl of permeabilized cells (2×10^6 to 2.5×10^6 cells) in a final volume of 125 μl. One EU was defined as 1 nmol of [*adenine*-2,8-^{3}H]ADP-ribose incorporated into the acid-insoluble material in 10 min at 37°C.

Proliferation assay. Assays were performed in flat-bottommed 96-well microplates (Falcon 3072) by incubating 5000 cells per well in a total volume of 200 μl at 37°C in a CO_2 atmosphere. Twenty microliters of [^{3}H]-TdR (specific activity 5 Ci/mM; Amersham, Buckinghamshire, England) were added to each well. Cells were harvested 1 h later onto glass fiber strips with an automatic cell harvester (Titertek 530; Flow Laboratories, Les Ulis, France). The incorporation of [^{3}H]-TdR measured by a beta counter (Wallac 1409; Pharmacia, Turku, Finland) was expressed in counts/minute. The results were calculated as the mean ± SD of quadruplicate samples and generally expressed in percent of control, untreated cells. Each experiment was repeated at least four times.

MTT assay. This assay was performed essentially according to [13], with minor modifications. Briefly, cells were seeded at 25×10^3/ml, in 24-well plates (Falcon 3047; Becton Dickinson, Le-Pont-De-Claix, France), and treated with 6(5H)-phenanthridinone and chloromethine as indicated above. Each experimental assay was performed in duplicate. After 4 days of incubation, cells were harvested, centrifuged for 10 min at 800 *g*, and resuspended in 500 μl. Four wells per assay of flat-bottommed 96-well plates were filled with 100 μl of the suspensions, and 20 μl of a MTT solution (1 mg/ml; Sigma Chemical Co., St Louis, MO, USA) were added. The plates were then incubated for 4 h at 37°C. To solubilize the formazan crystals, 100 μl of isopropanol in HCl 0.04 N was added, and the content of each well was vigorously mixed. Plates were read 30 min later at 570 nm on a microplate Flow Multiscan reader, and absorbance values from treated cells were compared with those of untreated control.

RESULTS

6(5H)-phenanthridinone inhibits PARP activity of whole cells. The ability of 6(5H)-phenanthridinone to block PARP activity was first demonstrated using purified enzyme [10]. To determine whether this compound was capable of inhibiting the poly(ADP-ribose) polymerase activity in whole cells, RDM4 tumor cells were incubated with 5 and 10 μM of 6(5H)-phenanthridinone for 24 h. RDM4 cells were then washed twice to eliminate the molecules of inhibitor not incorporated by the cells. Their PARP activity was then measured as described in Material and Methods. The same protocol was used with 3-MBA, the more potent classical PARP inhibitor, to compare their respective PARP inhibition potency. It appears that concentrations required to obtain 77% inhibition of the enzyme activity were about 500-fold lower in the case of 6(5H)-phenanthridinone as compared with 3-MBA (Figure 1).

Effect of 6(5H)-phenanthridinone on RDM4 viability and proliferation. We next evaluated the effects of 6(5H)-phenanthridinone on RDM4 viability and proliferation. RDM4 cells were cultured in the presence of 6(5H)-phenanthridinone at various concentrations. Cell number and viability were assessed. Even after several days of culture with the highest concentration (50 μM) of 6(5H)-phenanthridinone, we observed no toxicity of the compound. Indeed, the number of dead cells never exceeded 5%, a value not significantly different from untreated control cells (data not shown). Furthermore, no morphological changes in RDM4 cells were evident after this treatment.

For assessment of proliferation, the [^{3}H]-TdR incorporation assay was selected. Using this test, we found the IC_{50} value to be 25 μM after 24 h of treatment. This effect was concentration dependent (Figure 2). We also cultured RDM4 cells with 50 μM 6(5H)-phenanthridinone for 2 days, washed them twice, and placed them in fresh, 6(5H)-phenanthridinone-free medium. Under these conditions, cells totally recovered their proliferation rate (not shown). Thus, it appears that this isoquinoline derivative acts essentially as a cytostatic agent and that it is unable to induce cell death by itself.

Synergy of 6(5H)-phenanthridinone with chloromethine. Nitrogen mustards kill cells principally by introducing massive single- or double-strand breaks in DNA in the same way as γ rays and several other classes of anticancer drugs used in chemotherapy. As indicated above, PARP inhibitors have been frequently associated

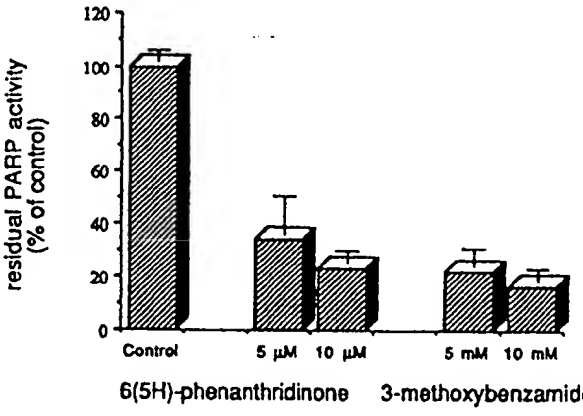

Figure 1. Inhibition of PARP activity in RDM4: comparison between 6(5H)-phenanthridinone and 3-MBA. Cells were seeded at 1×10^6/ml and incubated for 24 h at 37°C in the presence of 6(5H)-phenanthridinone (5 and 10 μM) or 3-MBA (5 and 10 mM). They were then harvested and washed twice with sucrose 0.32 M before determination of PARP activity, as indicated in Material and Methods. Results are expressed as the percent of control, and each assay was performed in triplicate. Bars represent SD.

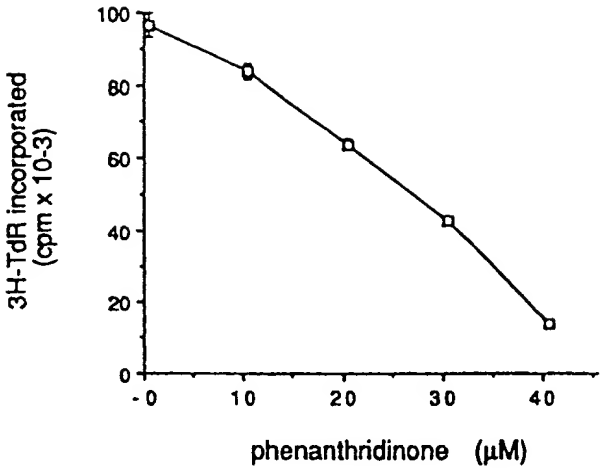

Figure 2. Effect of 6(5H)-phenanthridinone on RDM4 proliferation. RDM4 cells were cultured for different times in 96-well microplates, and [^{3}H]-TdR was added to each well. After 1 h of incubation, cells were harvested and the incorporation of [^{3}H]-TdR determined by liquid scintillation counting. Twenty-four hours after the onset of the treatment, a concentration-dependent inhibition of proliferation is recorded. Each point is the mean ± SD (bars) of quadruplicate determinations.

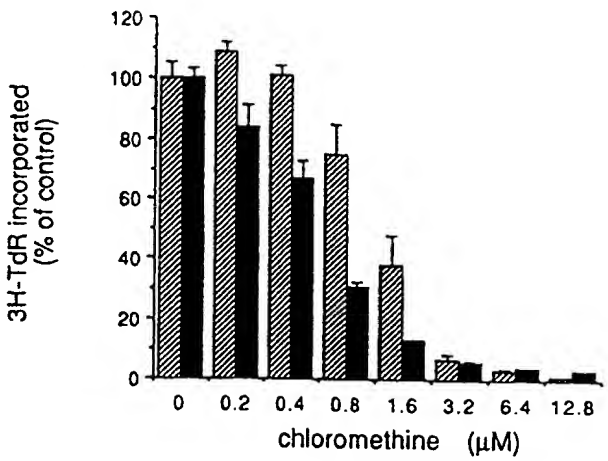

Figure 3. Effect of a treatment associating 6(5H)-phenanthridinone and chloromethine on RDM4 proliferation. Cells were cultured for 24 h in the presence of various concentrations of chloromethine and in the absence (hatched bars) or the presence (black bars) of 20 μM of 6(5H)-phenanthridinone. [^{3}H]-TdR was added for the last hour of culture. Results are expressed as the percent of control, untreated cells.

with anticancer drugs in experimental protocols to obtain a synergistic effect or at least a potentiation of their cytotoxicity by preventing DNA repair to occur [15]. Thus, it was of interest to look for the consequence of a combined treatment associating a DNA-damaging agent with 6(5H)-phenanthridinone. To investigate this point, RDM4 cells were treated with chloromethine. Preliminary experiments were carried out to determine its active concentrations on RDM4 cells. As expected, these lymphoma cells were found to be very sensitive to the alkylating agent, since 24 h after the onset of the culture, their growth was drastically inhibited by concentrations as low as 3 μM (Figure 3). In further experiments, 6(5H)-phenanthridinone at 20 μM was added together with chloromethine. As shown in Figure 3, an amplified, chloromethine concentration-dependent decrease of cell proliferation was then obtained. For instance, cells treated with chloromethine at 0.8 μM exhibit a 20% decrease in their [^{3}H]-TdR incorporation. This decrease rises to 70% when the cells are simultaneously treated with 6(5H)-phenanthridinone. We also determined whether cells treated simultaneously with the two compounds could resume proliferation 4 days after the beginning of the treatment. Indeed, after several days of culture, we usually noted that the proliferation potential of surviving cells cultured in the presence of the alkylating agent alone was progressively restored.

As shown in Table 1, which displays the [^{3}H]-TdR incorporation of a 4-day culture, cells treated with chloromethine alone rapidly recover a high proliferation rate. Indeed, for the 1.6 μM chloromethine-treated cells, the level of [^{3}H]-TdR incorporation reaches 87% of the control value (untreated cells). In contrast, in the presence of 6(5H)-phenanthridinone at 20 μM and 30 μM, the incorporation of [^{3}H]-TdR that was 87% and 70% respectively in the absence of chloromethine, fell down to 36% and 4% respectively when cultured in presence of 1.6 μM chloromethine. So, cells cultured in the

Table 1. Effect of a 4-day treatment combining various concentrations of chloromethine and 6(5H)-phenanthridinone upon RDM4 cell proliferation as assessed by [^{3}H]-TdR incorporation. Cells were labelled with [^{3}H]-TdR for 1 h before harvesting. Each value is expressed in cpm and represents the mean ± SD of quadruplicate wells.

6(5H)-phenanthridinone / chloromethine	0 μM	10 μM	20 μM	30 μM
0 μM	142400 ± 1400	134600 ± 1400	123400 ± 4800	100300 ± 4800
0.2 μM	128800 ± 10600	139000 ± 8100	135800 ± 8900	87500 ± 7800
0.4 μM	125000 ± 2900	125000 ± 4300	119900 ± 900	62900 ± 3400
0.8 μM	142800 ± 11100	133800 ± 800	100900 ± 3900	40700 ± 3100
1.6 μM	124000 ± 6100	96500 ± 6600	50700 ± 5900	5100 ± 300
3.2 μM	32800 ± 2000	19500 ± 3300	4700 ± 200	1200 ± 400
6.4 μM	2000 ± 100	1500 ± 100	500 ± 100	300 ± 100
12.8 μM	200 ± 100	300 ± 43	200 ± 27	100 ± 32

presence of both compounds present a decreased proliferation rate that is never obtained by 6(5H)-phenanthridinone or chloromethine alone and that is too low to be ascribed to a cumulative effect of these two compounds. Therefore it can be concluded from these experiments that a persisting synergistic effect on RDM4 proliferation is obtained when 6(5H)-phenanthridinone and chloromethine are associated. These observations were confirmed by the MTT test (Figure 4).

DISCUSSION

The purpose of the present study was to evaluate the effects of 6(5H)-phenanthridinone, a potent, recently described poly(ADP-ribose) polymerase inhibitor, on tumor cells in culture and to investigate whether the sensitivity of the latter to a DNA-damaging agent, chloromethine, could be modified. The data demonstrate that (i) PARP activity in 6(5H)-phenanthridinone-treated RDM4 cells is inhibited at micromolar concentrations, (ii) proliferation is reduced, and (iii) a marked synergistic effect is achieved when cells are cotreated with the alkylating drug. These results therefore clearly demonstrate the potential of 6(5H)-phenanthridinone as a possible candidate in anticancer chemotherapy.

It is now accepted that PARP is a privileged target for anticancer treatments [14, 15]. Indeed, the inhibition of poly(ADP-ribosyl)ation generally leads to an increase in tumor-cell destruction, due to the impairment of DNA repair following damage caused by anticancer drugs [16, 17], radiation [18–20], or cytotoxic cytokines [21]. Treatments combining these agents and classical PARP inhibitors have been tested in cancer therapy for a long time by many investigators using essentially nicotinamide, 3-aminobenzamide, and 3-MBA as inhibitors.

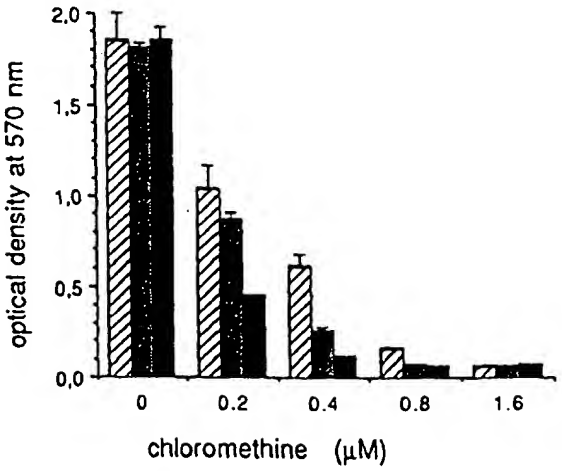

Figure 4. Cytotoxicity of chloromethine, in a combined treatment with 6(5H)-phenanthridinone, added simultaneously to RDM4 cell cultures. Viable cells number remaining 4 days after the beginning of the culture is assessed by the MTT colorimetric test. Hatched bars, chloromethine alone; grey bars, chloromethine plus 10 μM 6(5H)-phenanthridinone; black bars, chloromethine plus 20 μM 6(5H)-phenanthridinone.

However, in spite of encouraging results, there are to date no clinical applications in cancer therapy of such associations. It may be due to the high (millimolar) concentrations of inhibitors required for the PARP blocking to occur and also to their lack of specifity for this enzyme, since they act on several other metabolic pathways [22]. Other reasons can also be envisaged.

First, it must be noted that most attempts to reinforce the effect of chemotherapy with these compounds have been achieved using tumor cells in culture. Since we recently demonstrated [23] that endogenous PARP activity in tumor cells maintained in culture can be severalfold higher than in cells freshly recovered from ascitic or solid tumors, the role of PARP in tumor development should be reevaluated. Thus, the *in vivo* efficiency of such combined treatment remains to be established. Second, PARP activation following treatment with a DNA-damaging agent can vary according to the type of tumor cells used [24]; furthermore, the use of PARP inhibitors can lead to opposite effects. Indeed, in some cases, 3-aminobenzamide or nicotinamide can protect tumor cells from adriamycin-induced death [25]. Similarly, L929 fibroblastic cells become more resistant to the TNF-induced lysis, whereas SK-OV-3 neurologic tumor cells treated with the same inhibitors exhibit a higher sensitivity to this cytokine [21]. Third, the exact role of PARP on DNA breakage is far from totally elucidated [26]. Thus, it is clear that, to better evaluate these different points, more potent and specific PARP inhibitors are needed. Our data indicates that 6(5H)-phenanthridinone could well fulfill such a role. However, several questions remain to be elucidated concerning this compound. For instance, whereas benzamide derivatives used in most studies exert their action principally by mimicking the nicotinamide moiety of NAD, the mechanism by which isoquinoline derivatives block the enzyme is not known. Given their considerably higher activity and specificity, their sites of action could well be different from those of classical PARP inhibitors. Compounds belonging to this family are polyaromatic heterocyclics; the presence of a carbonyl group in the second ring conjugated with a six-membered aromatic group seems to constitute a common feature for this new class of inhibitors [10]. The position of the third aromatic ring also seems to be critical for the inhibition to occur, since acridone, a structural isomere of 6(5H)-phenanthridinone, was found to be unable to block PARP activity (data not shown).

In conclusion, 6(5H)-phenanthridinone, and possibly other related isoquinoline derivatives, constitutes a useful tool to explore the exact contribution of PARP in cancer development and therapy. Their use as adjunct in chemotherapy should also be seriously considered. Therefore, we are currently evaluating the effects of 6(5H)-phenanthridinone *in vivo* in normal mice and, in association with alkylating and intercalating agents, in tumor-transplanted mice.

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837 f2d 1071

In re David H. Fine

No. 87-1319

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

837 F.2d 1071; 1988 U.S. App. LEXIS 686; 5 U.S.P.Q.2D (BNA) 1596

January 26, 1988, Decided

PRIOR HISTORY:
[**1]

Appealed from: Board of Patent Appeals and Interferences of the United States Patent and Trademark Office.

COUNSEL:

Morris Relson, Darby & Darby, P.C., argued for Appellant. With him on the brief was Beverly B. Goodwin.

Lee E. Barrett, Associate Solicitor, Office of the Solicitor, argued for Appellee. With him on the brief were Joseph F. Nakamura, Solicitor and Fred E. McKelvey, Deputy Solicitor.

JUDGES:

Friedman, Smith, and Mayer, Circuit Judges. Smith, Circuit Judge, dissenting.

OPINIONBY:

MAYER

OPINION:

[*1072] MAYER, Circuit Judge.

David H. Fine appeals from a decision of the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office (Board) affirming the rejection of certain claims of his application, Serial No. 512,374, and concluding that his invention would have been obvious to one of ordinary skill in the art and was therefore unpatentable under *35 U.S.C. § 103.* We reverse.

Background

A. The Invention.

The invention claimed is a system for detecting and measuring minute quantities of nitrogen compounds. According to Fine, the system has the ability to detect the presence of nitrogen compounds in quantities [**2] as minute as one part in one billion, and is an effective means to detect drugs and explosives, which emanate nitrogen compound vapors even when they are concealed in luggage and closed containers.

The claimed invention has three major components: (1) a gas chromatograph which separates a gaseous sample into its constituent parts; (2) a converter which converts the nitrogen compound effluent output of the chromatograph into nitric oxide in a hot, oxygen-rich environment; and (3) a detector for measuring the level of nitric oxide. The claimed invention's sensitivity is achieved by combining nitric oxide with ozone to produce nitrogen dioxide which concurrently causes a detectable luminescence. The luminescence, which is measured by a visual detector, shows the level of nitric oxide which in turn is a measure of nitrogen compounds found in the sample.

The appealed claims were rejected by the Patent and Trademark Office (PTO) under *35 U.S.C. § 103.* Claims 60, 63, 77 and 80 were rejected as unpatentable over Eads, Patent No. 3,650,696 (Eads) in view of Warnick, et al., Patent No. 3,746,513 (Warnick). Claims 62, 68, 69, 79, 85 and 86 were rejected as unpatentable [**3] over Eads and Warnick in view of Glass, et al., Patent No. 3,207,585 (Glass).

B. The Prior Art.

1. *Eads Patent.*

Eads discloses a method for separating, identifying and quantitatively monitoring [*1073] sulfur

compounds. The Eads system is used primarily in "air pollution control work in the scientific characterization of odors from sulfur compounds."

The problem addressed by Eads is the tendency of sulfur compounds "to adhere to or react with the surface materials of the sampling and analytical equipment, and/or react with the liquid or gaseous materials in the equipment." Because of this, the accuracy of measurement is impaired. To solve the problem, the Eads system collects an air sample containing sulfur compounds in a sulfur-free methanol solution. The liquid is inserted into a gas chromatograph which separates the various sulfur compounds. The compounds are next sent through a pyrolysis furnace where they are oxidized to form sulfur dioxide. Finally, the sulfur dioxide passes through a measuring device called a microcoulometer which uses titration cells to calculate the concentration of sulfur compounds in the sample.

2. *Warnick Patent.*

Warnick [**4] is directed to a means for detecting the quantity of pollutants in the atmosphere. By measuring the chemiluminescence of the reaction between nitric oxide and ozone, the Warnick device can detect the concentration of nitric oxide in a sample gaseous mixture.

Warnick calls for "continuously flowing" a sample gaseous mixture and a reactant containing ozone into a reaction chamber. The chemiluminescence from the resulting reaction is transmitted through a light-transmitting element to produce continuous readouts of the total amount of nitric oxide present in the sample.

3. *Glass Patent.*

The invention disclosed in Glass is a device for "completely burning a measured amount of a substance and analyzing the combustion products." A fixed amount of a liquid petroleum sample and oxygen are supplied to a flame. The flame is then spark-ignited, causing the sample to burn. The resulting combustion products are then collected and measured, and from this measurement the hydrogen concentration in the sample is computed.

C. The Rejection.

The Examiner rejected claims 60, 63, 77 and 80 because "substitution of the [nitric oxide] detector of Warnick for the sulfur detector of Eads [**5] would be an obvious consideration if interested in nitrogen compounds, and would yield the claimed invention." He further asserted that "Eads teaches the [claimed] combination of chromatograph, combustion, and detection, in that order. ... Substitution of detectors to measure any component of interest is well within the skill of the art." In rejecting claims 62, 68, 69, 79, 85 and 86, the Examiner said, "Glass et al. teach a flame conversion means followed by a detector, and substitution of the flame conversion means of Glass et al. for the furnace of Eads would be an obvious equivalent and would yield the claimed invention." The Board affirmed the Examiner's rejection.

Discussion

A. Standard of Review.

Obviousness under *35 U.S.C. § 103* is "'a legal conclusion based on factual evidence.'" *Stratoflex, Inc. v. Aeroquip Corp., 713 F.2d 1530, 1535, 218 USPQ 871, 876 (Fed. Cir. 1983)* (quoting *Stevenson v. Int'l Trade Comm'n, 612 F.2d 546, 549, 204 USPQ 276, 279, 67 C.C.P.A. 109 (CCPA 1979)).* Therefore, an obviousness determination [**6] is not reviewed under the clearly erroneous standard applicable to fact findings, *Raytheon Co. v. Roper Corp., 724 F.2d 951, 956, 220 USPQ 592, 596 (Fed. Cir. 1983);* it is "reviewed for correctness or error as a matter of law." *In re De Blauwe, 736 F.2d 699, 703, 222 USPQ 191, 195 (Fed. Cir. 1984).*

To reach a proper conclusion under § 103, the decisionmaker must step backward in time and into the shoes worn by [a person having ordinary skill in the art] when the invention was unknown and just before it was made. In light of *all* the evidence, the decisionmaker must then determine whether ... the claimed invention as a whole would have been [*1074] obvious at *that* time to *that* person. *35 U.S.C. § 103.* The answer to that question partakes more of the nature of law than of fact, for it is an ultimate conclusion based on a foundation formed of all the probative facts.

Panduit Corp. v. Dennison Mfg. Co., 810 F.2d 1561, 1566, 1 USPQ2d 1593, 1595-96 (Fed. Cir. 1987).

B. Prima [**7] Facie Obviousness.

Fine says the PTO has not established a *prima facie* case of obviousness. He contends the references applied by the Board and Examiner were improperly combined, using hindsight reconstruction, without evidence to support the combination and in the face of contrary teachings in the prior art. He argues that the appealed claims were rejected because the PTO thought it would have been "obvious to try" the claimed invention, an unacceptable basis for rejection.

We agree. The PTO has the burden under section 103 to establish a *prima facie* case of obviousness. *See In re Piasecki, 745 F.2d 1468, 1471-72, 223 USPQ 785,*

787-87 (Fed. Cir. 1984). It can satisfy this burden only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references. *In re Lalu, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed. Cir. 1984); see also Ashland Oil, Inc. v. Delta Resins & Refractories, Inc., 776 F.2d 281, 297 n. 24, 227 USPQ 657, 667 n.24 (Fed. Cir. 1985);* [**8] *ACS Hosp. Sys., Inc. v. Montefiore Hosp., 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984).* This it has not done. The Board points to nothing in the cited references, either alone or in combination, suggesting or teaching Fine's invention.

The primary basis for the Board's affirmance of the Examiner's rejection was that it would have been obvious to substitute the Warnick nitric oxide detector for the Eads sulfur dioxide detector in the Eads system. The Board reiterated the Examiner's bald assertion that "substitution of one type of detector for another in the system of Eads would have been within the skill of the art," but neither of them offered any support for or explanation of this conclusion.

Eads is limited to the analysis of sulfur compounds. The particular problem addressed there is the difficulty of obtaining precise measurements of sulfur compounds because of the tendency of sulfur dioxide to adhere to or react with the sampling analytic equipment or the liquid or gaseous materials in the equipment. It solves this problem by suggesting that the gaseous sample containing sulfur compounds be absorbed into sulfur-free methanol and then inserted into [**9] a gas chromatograph to separate the sulfur compounds.

There is no suggestion in Eads, which focuses on the unique difficulties inherent in the measurement of sulfur, to use that arrangement to detect nitrogen compounds. In fact, Eads says that the presence of nitrogen is undesirable because the concentration of the titration cell components in the sulfur detector is adversely affected by substantial amounts of nitrogen compounds in the sample. So, instead of suggesting that the system be used to detect nitrogen compounds, Eads deliberately seeks to avoid them; it warns against rather than teaches Fine's invention. *See W. L. Gore & Assoc. v. Garlock, Inc., 721 F.2d 1540, 1550, 220 USPQ 303, 311 (Fed. Cir. 1983)* (error to find obviousness where references "diverge from and teach away from the invention at hand"). In the face of this, one skilled in the art would not be expected to combine a nitrogen-related detector with the Eads system. Accordingly, there is no suggestion to combine Eads and Warnick.

Likewise, the teachings of Warnick are inconsistent with the claimed invention, to some extent. The Warnick claims are directed to a gas stream from engine exhaust [**10] "continuously flowing the gaseous mixtures into the reaction chamber" to obtain "continuous readouts" of the amount of nitric oxide in the sample. In other words, it contemplates measuring the total amount of nitric oxide in a continuously flowing gaseous mixture of unseparated nitrogen constituents. By contrast, in Fine each [*1075] nitrogen compound constituent of the gaseous sample is retained in the chromatograph for an individual time period so that each exits in discrete, time-separated pulses. * By this process, each constituent may be both identified by its position in time sequence, and measured. The claimed system, therefore, diverges from Warnick and teaches advantages not appreciated or contemplated by it.

> * The Solicitor argues that the contents of Attachment C of Fine's brief were not before the Board and may not properly be considered here. However, we need not rely on Attachment C. It is merely illustrative of the qualitative separation of nitrogen compounds which occurs in Fine's system. The fact that the various constituents exit at discrete intervals is shown by the specification which was before the Board and which may appropriately be considered on appeal. *See, e.g., Astra-Sjuco, A.B. v. United States Int'l Trade Comm'n, 67 C.C.P.A. 128, 629 F.2d 682, 686, 207 USPQ 1, 5 (CCPA 1980)* (claims must be construed in light of specification).

[**11]

Because neither Warnick nor Eads, alone or in combination, suggests the claimed invention, the Board erred in affirming the Examiner's conclusion that it would have been obvious to substitute the Warnick nitric oxide detector for the Eads sulfur dioxide detector in the Eads system. *ACS Hosp. Sys., 732 F.2d at 1575-77, 221 USPQ at 931-33.* The Eads and Warnick references disclose, at most, that one skilled in the art might find it obvious to try the claimed invention. But whether a particular combination might be "obvious to try" is not a legitimate test of patentability. *In re Geiger, 815 F.2d 686, 688, 2 USPQ2d 1276, 1278 (Fed. Cir. 1987); In re Goodwin, 576 F.2d 375, 377, 198 USPQ 1, 3 (CCPA 1978).*

Obviousness is tested by "what the combined teachings of the references would have suggested to those of ordinary skill in the art." *In re Keller, 642 F.2d 413, 425, 208 USPQ 871, 881 (CCPA 1981).* But it "cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching [**12] or suggestion supporting the combination." *ACS Hosp. Sys., 732 F.2d at 1577, 221*

USPQ at 933. And "teachings of references can be combined *only* if there is some suggestion or incentive to do so." *Id.* Here, the prior art contains none.

Instead, the Examiner relies on hindsight in reaching his obviousness determination. But this court has said, "To imbue one of ordinary skill in the art with knowledge of the invention in suit, when no prior art reference or references of record convey or suggest that knowledge, is to fall victim to the insidious effect of a hindsight syndrome wherein that which only the inventor taught is used against its teacher." *W.L. Gore, 721 F.2d at 1553, 220 USPQ at 312-13.* It is essential that "the decisionmaker forget what he or she has been taught at trial about the claimed invention and cast the mind back to the time the invention was made ... to occupy the mind of one skilled in the art who is presented only with the references, and who is normally guided by the then-accepted wisdom in the art." *Id.* One cannot use [**13] hindsight reconstruction to pick and choose among isolated disclosures in the prior art to depreciate the claimed invention.

C. Advantage Not Appreciated by the Prior Art.

The Board erred not only in improperly combining the Eads and Warnick references but also in failing to appreciate that the appealed claims can be distinguished over that combination. A material limitation of the claimed system is that the conversion to nitric oxide occur in the range of 600 degrees C to 1700 degrees C. The purpose of this limitation is to prevent nitrogen from other sources, such as the air, from being converted to nitric oxide and thereby distorting the measurement of nitric oxide derived from the nitrogen compounds of the sample.

The claimed nitric oxide conversion temperature is not disclosed in Warnick. Although Eads describes a preferred temperature of 675 degrees C to 725 degrees C, the purpose of this range is different from that of Fine. Eads requires the 675 degrees C to 725 degrees C range because it affords a temperature low enough to avoid formation of unwanted sulfur trioxide, yet high enough to avoid formation of unwanted sulfides. Fine's temperature [*1076] range, in contrast, [**14] does not seek to avoid the formation of sulfur compounds or even nitrogen compounds. It enables the system to break down the nitrogen compounds of the sample while avoiding the destruction of background nitrogen gas. There is a partial overlap, of course, but this is mere happenstance. Because the purposes of the two temperature ranges are entirely unrelated, Eads does not teach use of the claimed range. *See In re Geiger, 815 F.2d at 688, 2 USPQ2d at 1278.* The Board erred by concluding otherwise.

D. Unexpected Results.

Because we reverse for failure to establish a *prima facie* case of obviousness, we need not reach Fine's contention that the Board failed to accord proper weight to the objective evidence of unexpected superior results. *Id.*

E. The "Flame" Claims.

Claims 62, 68, 69, 79, 85 and 86 relate to the oxygen-rich flame conversion means of the claimed invention. These "flame" claims depend from either apparatus claim 60 or method claim 77. Dependent claims are nonobvious under section 103 if the independent claims from which they depend are nonobvious. [**15] *Hartness Int'l, Inc. v. Simplimatic Eng'g Co., 819 F.2d 1100, 1108, 2 USPQ2d 1826, 1831 (Fed. Cir. 1987); In re Abele, 684 F.2d 902, 910, 214 USPQ 682, 689 (CCPA 1982); see also In re Sernaker, 702 F.2d 989, 991, 217 USPQ 1, 3 (Fed. Cir. 1983).* In view of our conclusion that claims 60 and 77 are nonobvious, the dependent "flame" claims are also patentable.

Conclusion

The Board's decision affirming the Examiner's rejection of claims 60, 62, 63, 68, 69, 77, 79, 80, 85 and 86 of Fine's application as unpatentable over the prior art under *35 U.S.C. § 103* is

REVERSED.

DISSENTBY:
SMITH

DISSENT:

SMITH, Circuit Judge, dissenting.

I respectfully dissent. I am of the firm belief that the prior art references, relied upon by the PTO to establish its prima facie case of obviousness, in combination teach and suggest Fine's invention to one skilled in the art. Also, I firmly believe that Fine failed to rebut the PTO's prima facie case. On this basis, I would affirm the board's determination sustaining the examiner's rejection, pursuant to *35 U.S.C. § 103,* of Fine's claims on appeal before [**16] this court.

2 uspq2d 1788

Ex parte James R. Skinner

Appeal No. 650-69 from Art Unit 138.

Application for Patent filed September 29, 1982, Serial No. 427,717. Mold, Molding Method And Molded Article.

Board of Patent Appeals and Interferences

1986 Pat. App. LEXIS 4; 2 U.S.P.Q.2D (BNA) 1788

November 25, 1986, Decided

[*1]

Before Winters, Goolkasian, and Emery, Examiners-in-Chief.

COUNSEL:

Hubert E. Dubb et al. for appellant.
Fliesler, Dubb, Meyer and Lovejoy
Four Embarcadero Center, Ste. 1740
San Francisco, CA 94111

Primary Examiner - Jay H. Woo.

OPINIONBY: GOOLKASIAN

OPINION:

Goolkasian, Examiner-in-Chief.

This is an appeal from the examiner's final rejection of claims 7 through 13, 20, and 21. Claims 1 through 6 and 14 through 19 remain in the case but stand withdrawn pursuant to a restriction requirement.

Appellant's invention is directed to a mold of the type used to produce plastic articles. The claimed mold is very smooth and is characterized by having a surface portion having a surface roughness of no more than about 12.5 X 10<-8> meters, RMS (root mean square). The mold surface is coated with a material which is substantially void-free, non-corroding, and has a Rockwell C hardness above about 60. This is achieved by vacuum deposition or sputtering of chromium or rhodium onto the mold surface. Claim 7 is illustrative and reads as follows:

7. In a mold (24) useful for preparing a molded plastic article (10), the mold (24) having a mold surface (30) having a surface portion (32) which is replicated onto the molded [*2] plastic article (10), an improvement comprising:

wherein said surface portion (32) has a surface roughness of no more than about 12.5 X 10<-8> meters, RMS; and including

a surface coating (34) covering said surface portion (32), said coating (34) being substantially void-free, substantially non-corroding when exposed to ambient atmospheric conditions, having a Rockwell C hardness above about 60 and having a surface roughness of no more than about 12.5 X 10<-8> meters, RMS.

The references relied on by the examiner are:

Mizutani et al. (Mizutani)	4,138,086	Feb. 06, 1979

Nyman et al. (Nyman)	4,262,875	Apr. 21, 1981
Japan	54-25285	Feb. 26, 1979

Claims 7, 8, 11, and 12 stand rejected under *35 U.S.C. 102* over Mizutani. Claims 7 through 13, 20, and 21 stand rejected under *35 U.S.C. 103* over Mizutani in view of Nyman and further in view of Japanese Patent Pub. No. 54-25285.

We consider first the rejection under *35 U.S.C. 102.* The Mizutani reference is directed to a mold used for manufacturing contact lenses. The mold is composed of two metallic mold halves forming a cavity in which a silicone resin contact lens may be molded. The surface of each section of the mold [*3] is plated with chromium or nickel so that a contact lens having high surface optical quality can be manufactured. The mold is said to be plated with chromium or nickel and is said to impart excellent optical property to the surface of the lens. It is the examiner's position, as stated in the Final Rejection, that "[a]lthough the patentee does not explicitly disclose the properties claimed by applicant, such properties may be inherent characteristics of the reference coating." (Emphasis added.) * We reverse.

* The quoted language appears in the office action mailed 11/22/83 (paper no. 5) which is expressly referred to in the Final Rejection.

It is by now well settled that the burden of establishing a prima facie case of anticipation resides with the Patent and Trademark Office. *In re Piasecki, 745 F.2d 1468, 1472, 223 USPQ 785, 788 (Fed. Cir. 1984)* quoting *In re Warner, 379 F.2d 1011, 1016, 154 USPQ 173, 177 (CCPA 1967).* It is the examiner's position that the mold of Mizutani may inherently have the characteristics of the claimed mold. Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing [*4] may result from a given set of circumstances is not sufficient. *In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323 (CCPA 1981).* We are mindful that there is a line of cases represented by *In re Swinehart, 439 F.2d 210, 169 USPQ 226 (CCPA 1971)* which indicates that where an examiner has reason to believe that a functional limitation asserted to be critical for establishing novelty in the claimed subject matter may, in fact, be an inherent characteristic of the prior art, the examiner possesses the authority to require an applicant to prove that the subject matter shown to be in the prior art does not possess the characteristic relied on. Nevertheless, before an applicant can be put to this burdensome task, the examiner must provide some evidence or scientific reasoning to establish the reasonableness of the examiner's belief that the functional limitation is an inherent characteristic of the prior art. In the case before us, no such evidence or reasoning has been set forward.

Appellant urges that the mold for a contact lens would not reasonably be expected to have a surface roughness of no more than about 12.5 X 10<-8> meters, RMS. In this regard, we note that appellant's [*5] specification indicates that the desired degree of surface smoothness is only achieved by polishing or diamond turning of the surface finish. See page 2, lines 12 through 14. Moreover, appellant utilizes a sputtering technique to apply the chromium onto the mold surface rather than a plating technique as utilized by the reference patent. Absent reasons on the part of the examiner regarding why the natural result of the process used to prepare the mold of Mizutani would have been to achieve the characteristics claimed by appellant's mold, a prima facie case of anticipation has not been established. See *In re Oelrich, supra.*

Claims 7 through 13, 20, and 21 stand rejected under *35 U.S.C. 103* over Mizutani in view of Nyman and further in view of the Japanese reference. We have carefully considered all of appellant's arguments but are unpersuaded of error in the examiner's rejection with respect to claims 7 through 13.

At the outset we note that appellant's specification indicates, as background, that it is known that molded plastic articles can be made with relatively smooth surfaces by providing a relatively smooth surface for the mold in which the article is manufactured. [*6] Relatively smooth surfaces are generally accomplished by machine grinding and lapping any portion of the mold surface which is to be replicated on a position on the plastic article where smoothness is desired. Appellant notes that surfaces having a roughness of 2.5 to 5 X 10<-8> meters, RMS, have been prepared in the art by polishing or diamond turning. We are in full agreement with the examiner that one of ordinary skill, desiring as smooth a surface as possible on the contact lens mold of Mizutani, would have considered it obvious to achieve a surface finish within the range claimed by appellant. The Nyman reference teaches quite clearly that a chromium layer on a mold serves to harden the surface of a mold and make it resistant to scratching of the surface by abrasive particles. We shall take official notice that chromium plated or chromium coated metal surfaces are more corrosion resistant than would be the base metal itself. Indeed, the Hackh's Chemical Dictionary definition of chromium describes chromium plating as "the electrolytic coating of metals with a layer of c.0.00001 in. thick over a layer of nickel which produces a

non-corrodible surface." The Nyman reference [*7] also teaches that plating of chromium onto mold surfaces develops a film which is "rough" (column 2, lines 64-66) and suggests vapor deposition techniques, e.g., sputtering and the like to apply a less rough, thin, stress free conformal layer containing chromium. One of ordinary skill having the references before him would have considered it obvious to improve the mold surface of the Mizutani mold by finely polishing the metal backing material to the required extent and then sputtering a layer of chromium thereover to improve the hardness and oxidation exposure of the base metal.

Claims 20 and 21, however, are directed to the use of rhodium as the metal in place of or in conjunction with chromium. The examiner relies on the Japanese reference for a teaching of utilizing a rhodium layer on the surface of a brass plate. The Japanese reference is directed to a method of making an ornamental part for a time piece (watch) by coating the part with a thin layer of compounds having a refractive index less than 2.41. Among the compounds which may be coated thereon are compounds of chromium, titanium, iron, copper, mercury, lead, and bismuth. Specifically, rhodium is first deposited * [*8] onto the brass base metal. Subsequently, a chromium layer and chromium oxide layer are vacuum deposited * thereon. The examiner has provided no information regarding why the Japanese reference used the rhodium coating as an undercoat and no reasons or incentive for utilizing the rhodium coating of the Japanese reference as an undercoat for the chromium of either Mizutani or Nyman.

* The Derwent translation of the Japanese reference indicates that the rhodium layer is deposited at three thousand angstrom thickness by a process described as "metallizing plating." While we would normally consider this to be an electroplating process, appellant's Brief (page 8) indicates that the rhodium is applied by vacuum deposition.

To properly combine the references to reach the conclusion that the subject matter of claims 20 and 21 would have been obvious, case law requires that there must have been some teaching, suggestion, or inference in either reference, or both, or knowledge generally available to one of ordinary skill in the relevant art, which would have led one of ordinary skill in the art to combine the relevant teachings of the references. See *ACS Hospital Systems, Inc. v.* [*9] *Montefiore Hospital, 732 F.2d 1572, 221 USPQ 929 (Fed. Cir. 1984).* When the incentive to combine the teachings of the references is not readily apparent, it is the duty of the examiner to explain why combination of the reference teachings is proper. In other words, the examiner must indicate the reasons why one skilled in the art would have substituted the sputtered chromium/rhodium combination of the Japanese patent for the sputtered chromium of the Nyman reference when it was substituted for the metal plated chromium of the Mizutani patent. Absent such reasons or incentives, the teachings of the references are not combinable. We reverse the examiner's rejection.

The examiner's rejection of claims 7, 8, 11, and 12 under *35 U.S.C. 102* is reversed. The examiner's rejection of claims 20 and 21 under *35 U.S.C. 103* is also reversed. The examiner's rejection of claims 7 through 13 under *35 U.S.C. 103* is affirmed.

AFFIRMED-IN-PART

927 f2d 1200

AMGEN, INC., Plaintiff/Cross-Appellant, v. CHUGAI PHARMACEUTICAL CO., LTD., and GENETICS INSTITUTE, INC., Defendants-Appellants

Nos. 90-1273, 90-1275

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

927 F.2d 1200; 1991 U.S. App. LEXIS 3481; 18 U.S.P.Q.2D (BNA) 1016

March 5, 1991, Decided

SUBSEQUENT HISTORY:

As Corrected April 2, 1991. Rehearing En Banc Declined May 20, 1991, Reported at: *1991 U.S. App. LEXIS 11131.*

PRIOR HISTORY:

[**1] Appealed from U.S. District Court for the District of Massachusetts; Judge Young.

DISPOSITION:

Affirmed- in- Part, Reversed-in-Part, Vacated-in-Part.

COUNSEL:

Edward M. O'Toole, Marshall, O'Toole, Gerstein, Murray & Bicknall, of Chicago, Illinois, argued for Plaintiff/Cross-Appellant. With him on the brief were Michael F. Borun, Richard A. Schnurr and Christine A. Dudzik. Also on the brief were Steven M. Odre and Robert D. Weist, Amgen, Inc., of Thousand Oaks, California, of Counsel.

Kurt E. Richter, Morgan & Finnegan, of New York, New York, and William F. Lee, Hale & Dorr, of Boston, Massachusetts, argued for Defendants-Appellants. Of Counsel were Eugene Moroz, Michael P. Dougherty and William S. Feiler, Morgan & Finnegan, of New York, New York.

JUDGES:

Markey, Lourie, and Clevenger, Circuit Judges.

OPINIONBY:

LOURIE

OPINION:

[*1202] LOURIE, Circuit Judge.

This appeal and cross appeal are from the March 4, 1990, judgment of the United States District Court for the District of Massachusetts, *Amgen, Inc. v. Chugai Pharmaceutical Co., 1989 U.S. Dist. LEXIS 16110, 13 U.S.P.Q.2d (BNA) 1737 (1990),* and involve issues of patent validity, infringement, and inequitable conduct with respect to two patents: U.S. Patent *4,703,008* (*'008*), owned by Kirin-Amgen [**2] Inc. (Amgen), and U.S. Patent *4,677,195* (*'195*), owned by Genetics Institute, Inc. (GI).

[*1203] Chugai Pharmaceutical Co., Ltd. (Chugai) and Genetics Institute, Inc. (collectively defendants) assert on appeal that the district court erred in holding that: 1) Amgen's *'008* patent is not invalid under *35 U.S.C. § § 102*(g) and 103; 2) the *'008* patent is enforceable; 3) the failure of Amgen to deposit the best mode host cells was not a violation of the best mode requirement under *35 U.S.C. § 112;* and 4) claims 4 and 6 of GI's *'195* patent are invalid for indefiniteness under *35 U.S.C. § 112.*

On cross appeal, Amgen challenges the district court's holdings that: 1) claims 1 and 3 of the *'195* patent are enabled; 2) the *'195* patent is enforceable; 3) this is not an exceptional case warranting an award of attorney fees to Amgen; and 4) claims 7, 8, 23-27 and 29 of the *'008* patent are not enabled by the specification.

We affirm the district court's holdings in all respects, except that we reverse the court's ruling that claims 1 and 3 of the *'195* patent are enabled. We also vacate that part of the district court's judgment relating to infringement of those claims.

BACKGROUND n1

n1 The district court, in a detailed opinion, fully sets out the scientific and historical background relating to the patents at issue. *See Amgen, 13 U.S.P.Q.2d (BNA) at 1741-58.* Familiarity with that opinion is presumed.

[**3]

Erythropoietin (EPO) is a protein consisting of 165 amino acids which stimulates the production of red blood cells. It is therefore a useful therapeutic agent in the treatment of anemias or blood disorders characterized by low or defective bone marrow production of red blood cells.

The preparation of EPO products generally has been accomplished through the concentration and purification of urine from both healthy individuals and those exhibiting high EPO levels. A new technique for producing EPO is recombinant DNA technology in which EPO is produced from cell cultures into which genetically-engineered vectors containing the EPO gene have been introduced. The production of EPO by recombinant technology involves expressing an EPO gene through the same processes that occur in a natural cell.

THE PATENTS

On June 30, 1987, the United States Patent and Trademark Office (PTO) issued to Dr. Rodney Hewick U.S. Patent *4,677,195*, entitled "Method for the Purification of Erythropoietin and Erythropoietin Compositions" (the *'195* patent). The patent claims both homogeneous EPO and compositions thereof and a method for purifying human EPO using reverse phase high performance liquid chromatography. [**4] The method claims are not before us. The relevant claims of the *'195* patent are:

1. Homogeneous erythropoietin characterized by a molecular weight of about 34,000 daltons on SDS PAGE, movement as a single peak on reverse phase high performance liquid chromatography and a specific activity of at least 160,000 IU per absorbance unit at 280 nanometers.

*** 3. A pharmaceutical composition for the treatment of anemia comprising a therapeutically effective amount of the homogeneous erythropoietin of claim 1 in a pharmaceutically acceptable vehicle.

4. Homogeneous erythropoietin characterized by a molecular weight of about 34,000 daltons on SDS PAGE, movement as a single peak on reverse phase high performance liquid chromatography and a specific activity of at least about 160,000 IU per absorbance unit at 280 nanometers.

*** 6. A pharmaceutical composition for the treatment of anemia comprising a therapeutically effective amount of the homogeneous erythropoietin of claim 4 in a pharmaceutically acceptable vehicle.

Dr. Hewick assigned the patent to GI.

The other patent in this litigation is U.S. Patent *4,703,008*, entitled "DNA Sequences Encoding Erythropoietin" (the *'008* patent), [**5] issued on October 27, 1987, to Dr. Fu-Kuen Lin, an employee of Amgen. The claims of [*1204] the *'008* patent cover purified and isolated DNA sequences encoding erythropoietin and host cells transformed or transfected with a DNA sequence. The relevant claims are as follows:

2. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding human erythropoietin.

*** 4. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to claim 1, 2 or 3 in a manner allowing the host cell to express erythropoietin.

*** 6. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to claim 5.

7. A purified and isolated DNA sequence consisting essentially of a DNA sequence encoding a polypeptide having an amino acid sequence sufficiently duplicative of that of erythropoietin to allow possession of the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake.

8. A cDNA sequence according to claim 7.

*** 23. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence [**6] according to claim 7, 8, or 11 in a manner allowing the host cell to express said polypeptide.

24. A transformed or transfected host cell according to claim 23 which host cell is capable of glycosylating said polypeptide.

25. A transformed or transfected mammalian host cell according to claim 24.

26. A transformed or transfected COS cell according to claim 25.

27. A transformed or transfected CHO cell according to claim 25.

*** 29. A procaryotic host cell stably transformed or transfected with a DNA vector according to claim 28.

PROCEDURAL HISTORY

On October 27, 1987, the same day that the *'008* patent was issued, Amgen filed suit against Chugai and GI. It alleged that GI infringed the *'008* patent by the production of recombinant EPO (rEPO) and by use of transformed mammalian host cells containing vectors with DNA coding for the production of human EPO, and that Chugai, as a result of a collaborative relationship with GI, had induced and/or contributed to the direct infringement of the *'008* patent by GI. Amgen further sought a declaration that GI's *'195* patent is invalid under *35 U.S.C. § § 102,* 103, and 112, or, in the alternative, that Amgen does not infringe the claims [**7] of the *'195* patent, and a declaration that GI and Chugai's future activities in the production and sale of rEPO will infringe the *'008* patent. n2

> n2 Amgen subsequently filed a complaint with the United States International Trade Commission alleging that Chugai's importation of rEPO, manufactured in Japan using genetically engineered host cells, violated Section 337 of the Tariff Act of 1930 *(19 U.S.C. § 1337,* 1337a). The Commission entered an order terminating the investigation for lack of subject matter jurisdiction. This court vacated and remanded, holding that the Commission should have treated the complaint on the merits and not on jurisdictional grounds, and that the claims of Amgen's patent did not cover a process for producing rEPO. *Amgen, Inc. v. United States Int'l Trade Comm'n, 902 F.2d 1532, 14 U.S.P.Q.2d (BNA) 1734 (Fed. Cir. 1990).*

GI and Chugai answered and counterclaimed, asserting several affirmative defenses, including invalidity under *35 U.S.C. § § 101,* 102, 103, and 112; non-infringement; failure to make [**8] deposits at a public depository of biological materials allegedly necessary for enabling the best mode of practicing the invention; and unenforceability of the patent because of Amgen's alleged inequitable conduct before the PTO. GI also counterclaimed, alleging that Amgen infringed the *'195* patent, asserting unfair competition, and seeking a declaratory judgment that the *'008* patent was invalid and not infringed.

GI and Chugai then filed a joint motion for a partial summary judgment that Amgen [*1205] infringed the claims of the *'195* patent. Chugai also filed its own motion for summary judgment. On February 24, 1988, the district court granted GI's and Chugai's motion for partial summary judgment and, on January 31, 1989, the court granted Chugai's motion for partial summary judgment only to the extent of ruling that the *'008* patent does not contain a process claim, an issue that is not now before us.

In response to Amgen's motion for a preliminary injunction, the district court, on February 7, 1989, issued an order finding that "Amgen had shown a reasonable likelihood of success on the merits of the validity of its patent; that it would suffer irreparable injury due to the needs of an incipient [**9] market and the attendant burdens on a new company; ..." and that, as to the public interest, "recombinant EPO is an extraordinarily valuable medicine that promises marked relief from renal failure." Because of this public interest finding, the court determined that it would not enter an order to delay or prevent production or shipping of EPO, but would require the defendant GI to place with the court all profits from the sale of EPO.

In order to expedite trial, the parties consented to trial before a magistrate. The judge entered judgment upon findings of fact and conclusions of law set forth by the magistrate. With respect to Amgen's *'008* patent, the court held that claims 2, 4, and 6 are valid, enforceable and have been infringed by GI; that infringement was not willful; that claims 7, 8, 23-27, and 29 are invalid for lack of enablement under *35 U.S.C. § 112* but, if valid, were infringed by GI; that the *'008* patent does not contain a process claim; and that Chugai has not infringed, contributorily infringed, or induced infringement of any claim of the *'008* patent. The court also dismissed Amgen's complaint against Chugai.

With respect to GI's *'195* patent, the court concluded that [**10] claims 1 and 3 are valid, enforceable, and have been infringed by Amgen; that Amgen has not infringed claims 2 and 5; that Amgen's infringement was not willful; and that claims 4 and 6 are invalid for indefiniteness under *35 U.S.C. § 112,* but, if valid, were infringed by Amgen. The court also concluded that Amgen did not misuse the *'008* patent and that this was not an "exceptional" case under *35 U.S.C. § 285.*

DISCUSSION

I. AMGEN's *'008* PATENT (Lin)

A. *Alleged prior invention under 35 U.S.C. § 102(g)*

The first issue we review is whether the district court erred in finding that the claims directed to a purified and isolated DNA sequence encoding human EPO were not invalidated by the work of GI's Dr. Fritsch. Section 102(g) provides in relevant part that:

A person is entitled to a patent unless -- (g) before the applicant's invention thereof the invention was made ...

by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, [**11] from a time prior to conception by the other.

Defendants assert error in the district court's legal conclusion that in this case Lin's conception occurred simultaneously with reduction to practice. *See e.g., Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1376, 231 U.S.P.Q. (BNA) 81, 87 (Fed. Cir. 1986), cert. denied, 480 U.S. 947, 94 L. Ed. 2d 792, 107 S. Ct. 1606 (1987).* They claim that Fritsch was first to conceive a probing strategy of using two sets of fully-degenerate cDNA probes of two different regions of the EPO gene to screen a gDNA library, which was the strategy which the district court found eventually resulted in the successful identification and isolation of the EPO gene. Defendants further claim that Fritsch conceived this strategy in 1981, was diligent until he reduced the invention to practice in May of 1984, and thus should be held to be a § 102(g) prior [*1206] inventor over Lin, who reduced the invention to practice in September of 1983.

Conception is the "formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice." *Hybritech, 802 F.2d at 1376, 231 U.S.P.Q. at 87* (citing 1 *Robinson on Patents* [**12] 532 (1890)); *Coleman v. Dines, 754 F.2d 353, 359, 224 U.S.P.Q. (BNA) 857, 862 (Fed. Cir. 1985)* (citing *Gunter v. Stream, 573 F.2d 77, 80, 197 U.S.P.Q. (BNA) 482, 484 (CCPA 1978)).* Conception requires both the idea of the invention's structure and possession of an operative method of making it. *Oka v. Youssefyeh, 849 F.2d 581, 583, 7 U.S.P.Q.2d (BNA) 1169, 1171 (Fed. Cir. 1988).*

In some instances, an inventor is unable to establish a conception until he has reduced the invention to practice through a successful experiment. This situation results in a simultaneous conception and reduction to practice. *See* 3 D. Chisum, *Patents* § 10.04[5] (1990). We agree with the district court that that is what occurred in this case.

The invention recited in claim 2 is a "purified and isolated DNA sequence" encoding human EPO. The structure of this DNA sequence was unknown until 1983, when the gene was cloned by Lin; Fritsch was unaware of it until 1984. As Dr. Sadler, an expert for GI, testified in his deposition: "You have to clone it first to get the sequence." In order to design a set of degenerate probes, one of which will hybridize with a particular gene, the amino acid sequence, or a portion thereof, of [**13] the protein of interest must be known. Prior to 1983, the amino acid sequence for EPO was uncertain, and in some positions the sequence envisioned was incorrect. Thus, until Fritsch had a complete mental conception of a purified and isolated DNA sequence encoding EPO and a method for its preparation, in which the precise identity of the sequence is envisioned, or in terms of other characteristics sufficient to distinguish it from other genes, all he had was an objective to make an invention which he could not then adequately describe or define.

A gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. *See Oka, 849 F.2d at 583, 7 U.S.P.Q.2d at 1171.* Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, *e.g.*, encoding human [**14] erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred, *i.e.*, until after the gene has been isolated.

Fritsch had a goal of obtaining the isolated EPO gene, whatever its identity, and even had an idea of a possible method of obtaining it, but he did not conceive a purified and isolated DNA sequence encoding EPO and a viable method for obtaining it until after Lin. It is important to recognize that neither Fritsch nor Lin invented EPO or the EPO gene. The subject matter of claim 2 was the novel *purified and isolated* sequence which codes for EPO, and neither Fritsch nor Lin knew the structure or physical characteristics of it and had a viable method of obtaining that subject matter until it was actually obtained and characterized.

Defendants further argue that because the trial court found that the probing and screening method [**15] employed by Lin is what distinguished the invention of the *'008* patent over the prior art, Fritsch's strategy in 1981 had priority over Lin's use of that strategy. We disagree. The trial court found that Fritsch's alleged conception in 1981 of an approach that might result in cloning the gene was mere speculation. [*1207] Conception of a generalized approach for screening a

DNA library that might be used to identify and clone the EPO gene of then unknown constitution is not conception of a "purified and isolated DNA sequence" encoding human EPO. It is not "a definite and permanent idea of the complete and operative invention." Fritsch's conception of a process had to be sufficiently specific that one skilled in the relevant art would succeed in cloning the EPO gene. *See Coleman, 754 F.2d at 359, 224 U.S.P.Q. at 862.* Clearly, he did not have that conception because he did not know the structure of EPO or the EPO gene.

The record indicates that several companies, as well as Amgen and GI, were unsuccessful using Fritsch's approach. As the trial court correctly summarized:

> Given the utter lack of experience in probing genomic libraries with fully degenerate probes and the crudeness of the techniques [**16] available in 1981, it would have been mere speculation or at most a probable deduction from facts then known by Dr. Fritsch that his generalized approach would result in cloning the EPO gene.

13 U.S.P.Q.2d at 1760. As expert testimony from both sides indicated, success in cloning the EPO gene was not assured until the gene was in fact isolated and its sequence known. Based on the uncertainties of the method and lack of information concerning the amino acid sequence of the EPO protein, the trial court was correct in concluding that neither party had an adequate conception of the DNA sequence until reduction to practice had been achieved; Lin was first to accomplish that goal.

Defendants also argue that the court failed to consider that 1983, just prior to Lin's conception, was the relevant time for determining the completeness of Fritsch's conception, not 1981. However, the record shows that the court did consider what occurred in 1983. Moreover, Fritsch had no more of a conception in 1983 than he did in 1981, because he did not then know the sequence of the gene encoding EPO.

B. *Alleged obviousness of the inventions of claims 2, 4, and 6*

Claim 2, as noted above, recites a purified [**17] and isolated DNA sequence, and claims 4 and 6 are directed to host cells transformed with such a DNA sequence. The district court determined that claims 2, 4, and 6 are not invalid under *35 U.S.C. § 103,* concluding that the unique probing and screening method employed by Lin in isolating the EPO gene and the extensive effort required to employ that method made the invention nonobvious over the prior art. n3

> n3 We note that both the district court and the parties have focused on the obviousness of a process for making the EPO gene, despite the fact that it is products (genes and host cells) that are claimed in the patent, not processes. We have directed our attention accordingly, and do not consider independently whether the products would have been obvious aside from the alleged obviousness of a method of making them.

Obviousness under Section 103 is a question of law. *Panduit Corp. v. Dennison Mfg. Co., 810 F.2d 1561, 1568, 1 U.S.P.Q.2d (BNA) 1593, 1597* (Fed. Cir.), *cert. denied, 481 U.S. 1052, 95 L. Ed. 2d 843, 107 S. Ct. 2187 (1987).* The district court [**18] stated that one must inquire whether the prior art would have suggested to one of ordinary skill in the art that Lin's probing and screening method should be carried out and would have a reasonable expectation of success, viewed in light of the prior art. *See In re Dow Chemical Co., 837 F.2d 469, 473, 5 U.S.P.Q.2d (BNA) 1529, 1531 (Fed. Cir. 1988).* "Both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure." *Id.*

The district court specifically found that, as of 1983, none of the prior art references "suggests that the probing strategy of using two fully-redundant [sic] sets of probes, of relatively high degeneracy [sic], to screen a human genomic library would be likely to succeed in pulling out the gene of interest." n4 *13 U.S.P.Q.2d at 1768.* While [*1208] it found that defendants had shown that these procedures were "obvious to try," the references did not show that there was a reasonable expectation of success. *See In re O'Farrell, 853 F.2d 894, 903-04, 7 U.S.P.Q.2d (BNA) 1673, 1680-81 (Fed. Cir. 1988).*

> n4 At this point, some explanation of the involved technology may be useful, consistent with that expressed in the district court opinion. DNA consists of two complementary strands of nucleotides, which include the four basic compounds adenine(A), guanine(G), cytosine(C), and thymine(T), oriented so that bases from one strand weakly bond to the bases of the opposite strand. A bonds with T, and G bonds with C to form complementary base pairs. This bonding process is called hybridization and results in the formation of a stable duplex molecule. The

structure also includes 5-carbon sugar moieties with phosphate groups.

The genetic code for a particular protein depends upon sequential groupings of three nucleotides, called codons. Each codon codes for a particular amino acid. Since there are four nucleotide bases and three bases per codon, there are 64 (4x4x4) possible codons. Because there are only 20 natural amino acids, most amino acids are specified by more than one codon. This is referred to as a "redundancy" or "degeneracy" in the genetic code, a fact that complicates and renders more difficult the techniques of recombinant DNA.

In order to prepare a protein using recombinant DNA technology, the gene for the protein must first be isolated from a cell's total DNA by screening a library of that cell's DNA. The DNA library is screened by use of a probe, a synthetic radiolabelled nucleic acid sequence which can be used to detect and isolate complementary base sequences by hybridization. To design a probe when the gene has not yet been isolated, a scientist must know the amino acid sequence, or a portion thereof, of the protein of interest. Because some amino acids have several possible codons and the researcher cannot know which of the possible codons will actually code for an amino acid, he or she may decide to design a set of probes that covers all possible codons for each amino acid comprising the protein, known as a "fully-degenerate" set of probes. A library to be screened can be a genomic library (gDNA), which contains a set of all the DNA sequences found in an organism's cells or a complementary DNA (cDNA) library, which is much smaller and less complex than a gDNA library, and is used frequently when the tissue source for a given gene is known.

[**19]

Defendants challenge the district court's determination, arguing that, as of September 1983, one of ordinary skill in the art would have had a reasonable expectation of success in screening a gDNA library by Lin's method in order to obtain EPO. We agree with the district court's conclusion, which was supported by convincing testimony. One witness, Dr. Davies of Biogen, another biotechnology company that had worked on EPO, stated that he could not say whether Biogen scientists would have succeeded in isolating the EPO gene if Biogen had the EPO fragments that were available to Lin in 1983. Dr. Wall, a professor at UCLA, testified that it would have been "difficult" to find the gene in 1983, and that there would have been no more than a fifty percent chance of success. He said, "you couldn't be certain where in the genomic DNA your probe might fall." The court found that no one had successfully screened a genomic library using fully-degenerate probes of such high redundancy as the probes used by Lin. In the face of this and other evidence on both sides of the issue, it concluded that defendants had not shown by clear and convincing evidence that the procedures used by Lin would have [**20] been obvious in September 1983. We are not persuaded that the court erred in its decision.

Defendants assert that whether or not it would have been obvious to isolate the human EPO gene from a gDNA library with fully-degenerate probes is immaterial because it was obvious to use the already known monkey EPO gene as a probe. Defendants point out that, in the early 1980s, Biogen did significant work with an EPO cDNA obtained from a baboon, and that they used it as a probe to hybridize with the corresponding gene in a human gDNA library. However, this technique did not succeed until after Lin isolated the EPO gene with his fully-degenerate set of probes.

To support its obviousness assertion, defendants rely upon the testimony of their expert, Dr. Flavell, who testified that the overall homology of baboon DNA and human DNA was "roughly 90 percent". While this testimony indicates that it might have been feasible, perhaps obvious to try, to successfully probe a human gDNA library with a monkey cDNA probe, it does not indicate that the gene could have been identified and isolated with a reasonable likelihood of success. Neither the DNA nucleotide sequence of the human EPO gene nor its exact [**21] degree of homology with [*1209] the monkey EPO gene was known at the time.

Indeed, the district court found that Lin was unsuccessful at probing a human gDNA library with monkey cDNA until after he had isolated the EPO gene by using the fully-degenerate probes. Based on the evidence in the record, the district court found there was no reasonable expectation of success in obtaining the EPO gene by the method that Lin eventually used. While the idea of using the monkey gene to probe for a homologous human gene may have been obvious to try, the realization of that idea would not have been obvious. There were many pitfalls. Hindsight is not a justifiable basis on which to find that ultimate achievement of a long sought and difficult scientific goal was obvious. The district court thoroughly examined the evidence and the testimony. We see no error in its result. Moreover, if the DNA sequence was not obvious, host cells containing such sequence, as claimed in claims 4 and 6, could not have been obvious. We conclude that the district court

did not err in holding that the claims of the patent are not invalid under Section 103.

C. *Best Mode*

Defendants argue that the district court erred in failing [**22] to hold the *'008* patent invalid under *35 U.S.C. § 112,* asserting that Lin failed to disclose the best mammalian host cells known to him as of November 30, 1984, the date he filed his fourth patent application.

The district court found that the "best mode" of practicing the claimed invention was by use of a specific genetically-heterogeneous strain of Chinese hamster ovary (CHO) cells, which produced EPO at a rate greater than that of other cells. It further found that this strain was disclosed in Example 10 and that Lin knew of no better mode. GI argues that Lin's best mode was not adequately disclosed in Example 10 because one skilled in the art could not duplicate Lin's best mode without his having first deposited a sample of the specific cells in a public depository. The issue before us therefore is whether the district court erred in concluding that Example 10 of the *'008* patent satisfied the best mode requirement as to the invention of the challenged claims n5 and that a deposit of the preferred CHO cells was not necessary.

> n5 Defendants assert that all the claims should be invalid for failure to disclose the best mode. We perceive that the best mode issue only relates to the host cell claims, 4, 6, 23-27, and 29. Absent inequitable conduct, a best mode defense only affects those claims covering subject matter the practice of which has not been disclosed in compliance with the best mode requirement. *See Northern Telecom, Inc. v. Datapoint Corp., 908 F.2d 931, 940, 15 U.S.P.Q.2d (BNA) 1321, 1328* (Fed. Cir.), *cert. denied, 498 U.S. 920, 111 S. Ct. 296, 112 L. Ed. 2d 250 (1990).*

[**23]

A determination whether the best mode requirement is satisfied is a question of fact, *DeGeorge v. Bernier, 768 F.2d 1318, 1324, 226 U.S.P.Q. (BNA) 758, 763 (Fed. Cir. 1985);* we therefore review the district court's finding under a clearly erroneous standard.

35 U.S.C. § 112 provides in relevant part:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, *and shall set forth the best mode contemplated by the inventor of carrying out his invention.*

(Emphasis added).

This court has recently discussed the best mode requirement, pointing out that its analysis has two components. *Chemcast Corp. v. Arco Indus. Corp., 913 F.2d 923, 927, 16 U.S.P.Q.2d (BNA) 1033, 1036 (Fed. Cir. 1990).* The first is a subjective one, asking whether, at the time the inventor filed his patent application, he contemplated a best mode of practicing his invention. If he did, the second inquiry is whether his disclosure is adequate to enable one skilled in the art to practice [**24] the best mode or, in other words, whether the best mode has been concealed from the public. The best mode requirement thus is intended to ensure that a patent applicant [*1210] plays "fair and square" with the patent system. It is a requirement that the *quid pro quo* of the patent grant be satisfied. One must not receive the right to exclude others unless at the time of filing he has provided an adequate disclosure of the best mode known to him of carrying out his invention. Our case law has interpreted the best mode requirement to mean that there must be no concealment of a mode known by the inventor to be better than that which is disclosed. *Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384-85, 231 U.S.P.Q. (BNA) 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947, 94 L. Ed. 2d 792, 107 S. Ct. 1606 (1987).* Section 282 imposes on those attempting to prove invalidity the burden of proof. We agree that the district court did not err in finding that defendants have not met their burden of proving a best mode violation.

As noted above, the district court found that the best mode of making the CHO cells was set forth in Example 10. As the district court stated, while it was not clear which of two possible strains [**25] Lin considered to be the best, the cell strain subjected to 1000 nanomolar MTX (methotrexate) or that subjected to 100 nanomolar MTX, the best mode was disclosed because both were disclosed. n6 Defendants argue that this disclosure is not enough, that a deposit of the cells was required.

> n6 In its opinion, the district court stated that "the best way to express EPO was from mammalian cells ... and that a cell line derived from 11 possible clones from the CHO B11 3,.1 cell strain was to be used for Amgen's master working cell bank, which was expected to be started on November 26, 1984." *13 U.S.P.Q.2d at*

1772. At another point, the court stated that Amgen "did disclose the best mode in Example 10 of the invention, when it described the production rates of the 100 nanomolar-amplified cells (the B11 3,.1 cell strain) and one micromolar-treated cells." *Id.*

Defendants contend that "in the field of living materials such as microorganisms and cell cultures," we should require a biological deposit so that the public has access [**26] to exactly the best mode contemplated by the inventor. This presents us with a question of first impression concerning the best mode requirement for patents involving novel genetically-engineered biological subject matter.

For many years, it has been customary for patent applicants to place microorganism samples in a public depository when such a sample is necessary to carry out a claimed invention. This practice arose out of the development of antibiotics, when microorganisms obtained from soil samples uniquely synthesized antibiotics which could not be readily prepared chemically or otherwise. *In re Argoudelis, 58 C.C.P.A. 769, 434 F.2d 1390, 168 U.S.P.Q.(BNA) 99 (CCPA 1970).* Such a deposit has been considered adequate to satisfy the *enablement* requirement of *35 U.S.C. § 112,* when a written description alone would not place the invention in the hands of the public and physical possession of a unique biological material is required. *See, e.g., In re Wands, 858 F.2d 731, 735-36, 8 U.S.P.Q.2d (BNA) 1400, 1403 (Fed. Cir. 1988)* ("Where an invention depends on the use of living materials ... it may be impossible to enable the public to make the invention (*i.e.*, to obtain these living materials) solely [**27] by means of written disclosure."); *In re Lundak, 773 F.2d 1216, 1220, 227 U.S.P.Q. (BNA) 90, 93 (Fed. Cir. 1985)* ("When an invention relates to a new biological material, the material may not be reproducible even when detailed procedures and a complete taxonomic description are included in the specification."); *see generally* Hampar, *Patenting of Recombinant DNA Technology: The Deposit Requirement,* 67 J. Pat. & Trademark Off. Soc'y 569, 607 (1985) ("The deposit requirement is a nonstatutory mechanism for ensuring compliance with the 'enabling' provision under *35 U.S.C. § 112.*").

The district court found that the claims at issue require the use of biological materials that were capable of being prepared in the laboratory from readily available biological cells, using the description in Example 10. The court also found that there were no starting materials that were not publicly available, that were not described, or that required undue experimentation for their preparation in order to carry out the best mode. The court noted that Lin testified [*1211] that the isolation of the preferred strain was a "routine limited dilution cloning procedure[]" well known in the art. Dr. Simonsen, GI's own [**28] expert, testified that the disclosed procedures were "standard" and that:

with the vectors and the sequences shown in Example 10, I have no doubt that someone eventually could reproduce -- well, could generate cell lines [sic, strains] making some level of EPO, and they could be better, they could be worse in terms of EPO production.

The district court relied on this testimony, and, upon review, we agree with its determination. The testimony accurately reflects that the invention, as it relates to the *best mode* host cells, could be practiced by one skilled in the art following Example 10. Thus, the best mode was disclosed and it was adequately enabled.

These materials are therefore not analogous to the biological cells obtained from unique soil samples. When a biological sample required for the practice of an invention is obtained from nature, the invention may be incapable of being practiced without access to that organism. Hence the deposit is required in that case. On the other hand, when, as is the case here, the organism is created by insertion of genetic material into a cell obtained from generally available sources, then all that is required is a description of the best [**29] mode and an adequate description of the means of carrying out the invention, not deposit of the cells. If the cells can be prepared without undue experimentation from known materials, based on the description in the patent specification, a deposit is not required. *See Feldman v. Aunstrup, 517 F.2d 1351, 1354, 186 U.S.P.Q. (BNA) 108, 111 (CCPA 1975),* ("No problem exists when the microorganisms used are known and readily available to the public."), *cert. denied, 424 U.S. 912, 96 S. Ct. 1109, 47 L. Ed. 2d 316 (1976).* Since the court found that that is the case here, we therefore hold that there is no failure to comply with the best mode requirement for lack of a deposit of the CHO cells, when the *best mode* of preparing the cells has been disclosed and the best mode cells have been enabled, *i.e.*, they can be prepared by one skilled in the art from known materials using the description in the specification.

Defendants also contend that the examiner's rejection of the application that matured into the *'008* patent for failure to make a publicly accessible biological deposit supports its argument. U.S. Patent Application Serial No. 675,298, Prosecution History at 179 (First Rejection July 3, 1986). However, that rejection [**30]

was withdrawn after an oral interview and a written argument that the invention did not require a deposit. *Id.* at 208.

We also note that the PTO has recently prescribed guidelines concerning the deposit of biological materials. *See 37 C.F.R. § 1.802(b)* (1990) (biological material need not be deposited "if it is known and readily available to the public or can be made or isolated without undue experimentation"). The PTO, in response to a question as to whether the deposit requirement is applicable to the best mode requirement, as distinct from enablement, said:

> The best mode requirement is a safeguard against the possible selfish desire on the part of some people to obtain patent protection without making a full disclosure. The requirement does not permit an inventor to disclose only what is known to be the second-best embodiment, retaining the best .... The fundamental issue that should be addressed is whether there was evidence to show that the quality of an applicant's best mode disclosure is so poor as to effectively result in concealment. *In re Sherwood, 613 F.2d 809, 204 U.S.P.Q. (BNA) 537 (CCPA 1980).* If a deposit is the only way to comply with the best mode requirement [**31] then the deposit must be made.
>
> *52 Fed. Reg. 34080, 34086* (Sept. 8, 1987). n7
>
> n7 *See also 53 Fed. Reg. 39420, 39425* (Oct. 6, 1989) (comment *re* "deposit [to] satisfy the best mode requirement"); *52 Fed. Reg. 34080, 34080 and 34084* (Sept. 8, 1987) (deposit may be required to satisfy enablement, best mode, or distinct claim requirements of § 112).

We see no inconsistency between the district court's decision, which we affirm here, and these guidelines.

[*1212] Defendants also assert that the record shows that scientists were unable to duplicate Lin's genetically-heterogeneous best mode cell strain. However, we have long held that the issue is whether the disclosure is "adequate," not that an exact duplication is necessary. Indeed, the district court stated that

> the testimony is clear that no scientist could ever duplicate exactly the best mode used by Amgen, but that those of ordinary skill in the art could produce mammalian host cell strains or lines with similar levels of production identified in Example [**32] 10.

13 U.S.P.Q.2d at 1774. What is required is an adequate disclosure of the best mode, not a guarantee that every aspect of the specification be precisely and universally reproducible. *See In re Gay, 50 C.C.P.A. 725, 309 F.2d 769, 773, 135 U.S.P.Q. (BNA) 311, 316 (1962).*

Defendants finally argued that Lin's failure to deposit the transfected cells notwithstanding the fact that he was willing to deposit essentially worthless cell material was evidence of deliberate concealment. We have already stated that deposit of the host cells containing the rEPO gene was not necessary to satisfy the best mode requirement of Section 112. The best mode was disclosed and a deposit was not necessary to carry it out. Therefore, the fact that some cells were deposited, but not others, is irrelevant.

D. *Enablement of claims 7, 8, 23-27, and 29*

Amgen argues that the district court's holding that GI "provided clear and convincing evidence that the patent specification is insufficient to enable one of ordinary skill in the art to make and use the invention claimed in claim 7 of the *'008* patent without undue experimentation" constituted legal error. *13 U.S.P.Q.2d at 1776.* Amgen specifically argues that the district court erred [**33] because it "did not properly address the factors which this court has held must be considered in determining lack of enablement based on assertion of undue experimentation," citing this court's decision in *In re Wands, 858 F.2d at 737, 8 U.S.P.Q.2d at 1404.*

Claim 7 is a generic claim, covering all possible DNA sequences that will encode any polypeptide having an amino acid sequence "sufficiently duplicative" of EPO to possess the property of increasing production of red blood cells. As claims 8, 23-27, and 29, dependent on claim 7, are not separately argued, and are of similar scope, they stand or fall with claim 7. *See In re Dillon, 919 F.2d 688, 692, 16 U.S.P.Q.2d (BNA) 1897, 1900 (Fed. Cir. 1990)* (in banc).

Whether a claimed invention is enabled under *35 U.S.C. § 112* is a question of law, which we review *de novo. Moleculon Research Corp. v. CBS, Inc., 793 F.2d 1261, 1268, 229 U.S.P.Q. (BNA) 805, 811 (Fed. Cir. 1986), cert. denied, 479 U.S. 1030, 93 L. Ed. 2d 829, 107 S. Ct. 875 (1987).* "To be enabling under § 112, a patent must contain a description that enables one skilled in the art to make and use the claimed invention." *Atlas Powder Co. v. E.I. duPont De Nemours & Co., 750 F.2d 1569, 1576, 224 U.S.P.Q. (BNA) 409, 413 (Fed.* [**34] *Cir. 1984).*

That some experimentation is necessary does not constitute a lack of enablement; the amount of

experimentation, however, must not be unduly extensive. *Id.* The essential question here is whether the scope of enablement of claim 7 is as broad as the scope of the claim. *See generally In re Fisher, 57 C.C.P.A. 1099, 427 F.2d 833, 166 U.S.P.Q. (BNA) 18 (CCPA 1970);* 2 D. Chisum, *Patents* § 7.03[7][b] (1990).

The specification of the *'008* patent provides that:

one may readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for mature EPO in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions).

*** DNA sequences provided by the present invention are thus seen to comprehend all DNA sequences suitable for use in securing expression in a procaryotic [*1213] or eucaryotic host cell of a polypeptide product having at least a part of the primary structural conformation and one or more of the biological properties of erythropoietin, and selected from among: (a) the DNA sequences set out in FIGS. 5 and 6; (b) DNA sequences which hybridize [**35] to the DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

The district court found that over 3,600 different EPO analogs can be made by substituting at only a single amino acid position, and over a million different analogs can be made by substituting three amino acids. The patent indicates that it embraces means for preparation of "numerous" polypeptide analogs of EPO. Thus, the number of claimed DNA encoding sequences that can produce an EPO-like product is potentially enormous.

In a deposition, Dr. Elliott, who was head of Amgen's EPO analog program, testified that he did not know whether the fifty to eighty EPO analogs Amgen had made "had the biological property of causing bone marrow cells to increase production of reticulocytes and red blood cells, and to increase hemoglobin synthesis or iron uptake." Based on this evidence, the trial court concluded that "defendants had provided clear and convincing evidence that the patent specification is insufficient to enable one of ordinary skill in the art to make and use the invention claimed in claim [**36] 7 of the *'008* patent without undue experimentation." *13 U.S.P.Q. at 1776.* In making this determination, the court relied in particular on the lack of predictability in the art, as demonstrated by the testimony of both Dr. Goldwasser, another scientist who worked on procedures for purifying urinary EPO (uEPO), and Dr. Elliott. After five years of experimentation, the court noted, "Amgen is still unable to specify which analogs have the biological properties set forth in claim 7." *Id.*

We believe the trial court arrived at the correct decision, although for the wrong reason. By focusing on the biological properties of the EPO analogs, it failed to consider the enablement of the DNA sequence analogs, which are the subject of claim 7. Moreover, it is not necessary that a patent applicant test all the embodiments of his invention, *In re Angstadt, 537 F.2d 498, 502, 190 U.S.P.Q. (BNA) 214, 218 (CCPA 1976);* what is necessary is that he provide a disclosure sufficient to enable one skilled in the art to carry out the invention commensurate with the scope of his claims. For DNA sequences, that means disclosing how to make and use enough sequences to justify grant of the claims sought. Amgen has not [**37] done that here. In addition, it is not necessary that a court review all the *Wands* factors to find a disclosure enabling. They are illustrative, not mandatory. What is relevant depends on the facts, and the facts here are that Amgen has not enabled preparation of DNA sequences sufficient to support its all-encompassing claims.

It is well established that a patent applicant is entitled to claim his invention generically, when he describes it sufficiently to meet the requirements of Section 112. *See Utter v. Hiraga, 845 F.2d 993, 998, 6 U.S.P.Q.2d (BNA) 1709, 1714 (Fed. Cir. 1988)* ("A specification may, within the meaning of *35 U.S.C. § 112* para. 1, contain a written description of a broadly claimed invention without describing all species that claim encompasses."); *In re Robins, 57 C.C.P.A. 1321, 429 F.2d 452, 456-57, 166 U.S.P.Q. (BNA) 552, 555 (CCPA 1970)* ("Representative samples are not required by the statute and are not an end in themselves."). Here, however, despite extensive statements in the specification concerning all the analogs of the EPO gene that can be made, there is little enabling disclosure of particular analogs and how to make them. Details for preparing only a few EPO analog genes are disclosed. [**38] Amgen argues that this is sufficient to support its claims; we disagree. This "disclosure" might well justify a generic claim encompassing these and similar analogs, but it represents inadequate support for Amgen's desire to claim all EPO gene analogs. There may be many other genetic sequences that code for EPO-type products. Amgen has told how to [*1214] make and use only a few of them and is therefore not entitled to claim all of them.

In affirming the district court's invalidation of claims 7, 8, 23-27, and 29 under Section 112, we do not intend to imply that generic claims to genetic sequences cannot

be valid where they are of a scope appropriate to the invention disclosed by an applicant. That is not the case here, where Amgen has claimed every possible analog of a gene containing about 4,000 nucleotides, with a disclosure only of how to make EPO and a very few analogs.

The district court properly relied upon *Fisher* n8 in making its decision. In that case, an applicant was attempting to claim an adrenocorticotrophic hormone preparation containing a polypeptide having at least twenty-four amino acids of a specified sequence. Only a thirty-nine amino acid product was disclosed. The [**39] court found that applicant could not obtain claims that are insufficiently supported and hence not in compliance with the first paragraph of *35 U.S.C. § 112.* It stated:

Appellant's parent application, therefore, discloses no products, inherently or expressly, containing other than 39 amino acids, yet the claim includes all polypeptides, of the recited potency and purity, having at least 24 amino acids in the chain in the recited sequence. The parent specification does not enable one skilled in the art to make or obtain ACTHs with other than 39 amino acids in the chain, and there has been no showing that one of ordinary skill would have known how to make or obtain such other ACTHs without undue experimentation. As for appellant's conclusion that the 25th to 39th acids in the chain are unnecessary, it is one thing to make such a statement when persons skilled in the art are able to make or obtain ACTH having other than 39 amino acids; it is quite another thing when they are not able to do so. In the latter situation, the statement is in no way "enabling" and hence lends no further support for the broad claim. We conclude that appellant's parent application is insufficient to support [**40] a claim as broad as claim 4.

*** [Section 112] requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art.

Fisher, 427 F.2d at 836, 839, 166 U.S.P.Q. at 21-22, 24.

n8 *Cf. Hormone Research Foundation, Inc. v. Genentech, Inc., 904 F.2d 1558, 15 U.S.P.Q.2d (BNA) 1039 (Fed. Cir. 1990).* In *Hormone Research*, this court, in a remand, directed the district court to consider the effect of *United States Steel Corp. v. Phillips Petroleum Co., 865 F.2d 1247, 9 U.S.P.Q.2d (BNA) 1461 (Fed. Cir. 1989)* and *In re Hogan, 559 F.2d 595, 194 U.S.P.Q. (BNA) 527 (CCPA 1977)* on *Fisher* in its enablement analysis. The facts of our case are distinguishable from those in *Hormone Research, United States Steel*, and *Hogan.*

Considering the structural complexity of the EPO gene, the manifold possibilities for change in its structure, with attendant uncertainty as to what utility will be possessed by these analogs, we consider that more [**41] is needed concerning identifying the various analogs that are within the scope of the claim, methods for making them, and structural requirements for producing compounds with EPO-like activity. It is not sufficient, having made the gene and a handful of analogs whose activity has not been clearly ascertained, to claim all possible genetic sequences that have EPO-like activity. Under the circumstances, we find no error in the court's conclusion that the generic DNA sequence claims are invalid under Section 112.

E. *Inequitable Conduct*

Defendants argue that the *'008* patent claims are unenforceable as a result of an asserted misrepresentation of the number of probes Lin used for the monkey gene cloning described in Example 3 of his patent. Relying on the district court's finding that Lin had said that a "full set" mixture of 128 "EpV" probes n9 was used for monkey cDNA screening, whereas only a 16-member "subset" of the EpV mixture was actually used, defendants argue that the [*1215] court ought to have found that the representations were material.

n9 The probes designated "EpV" were from EPO amino acid sequence region 46-52.

[**42]

The essential elements of proof of inequitable conduct include intent to deceive and materiality. After finding threshold levels of materiality and intent, the trial court must balance the two and determine, in its discretion, whether inequitable conduct has occurred. *J.P. Stevens & Co. v. Lex Tex Ltd., Inc., 747 F.2d 1553, 1560, 223 U.S.P.Q. (BNA) 1089, 1092 (Fed. Cir. 1984), cert. denied, 474 U.S. 822, 88 L. Ed. 2d 60, 106 S. Ct. 73 (1985).* While we review an ultimate conclusion of inequitable conduct under an abuse of discretion standard, *Kingsdown Medical Consultants, Ltd. v. Hollister Inc., 863 F.2d 867, 876, 9 U.S.P.Q.2d (BNA) 1384, 1392 (Fed. Cir. 1988)* (in banc), *cert. denied, 490 U.S. 1067, 104 L. Ed. 2d 633, 109 S. Ct. 2068 (1989),* the underlying factual threshold findings are reviewed under a clearly erroneous standard.

Lin set out to clone the EPO gene by more than one method, including using degenerate human probes and monkey probes. It is not disputed that he did isolate the human EPO gene from a genomic library using two different 128-member pools of probes made from fragments of the human EPO protein. Thereafter, he also attempted to use the human sequence probes to find the monkey EPO cDNA to be used later as a probe to hybridize with [**43] the human EPO gene. Example 3 of the *'008* patent describes this work, indicating that the screening yielded seven positive clones. It also reports that a subset of the human EpV mixture was used for DNA sequencing work. When Lin published his monkey cDNA cloning work in a scientific journal, he also reported the use of 128 EpV probes to screen the monkey library. Lin screened the monkey library with the full mixture of 128 EpV probes and with one of eight subsets of probes which made up the full EpV mixture. In response to a question whether a subset of EpV probes was used in the first screening of the monkey cDNA library, Lin testified:

I don't know which we used, the subset first or used the full set first. I cannot recall exactly. It looks like the subset was first defining the number, yes.

This answer constituted the sole basis for the court's finding that, "at trial, Lin admitted he only used a subset of the EpV 128 probes in screening the cDNA library." *13 U.S.P.Q.2d at 1778.*

We consider that the district court's finding of an "admission" of misrepresentation in Lin's testimony and its conclusion that GI "presented clear and convincing evidence of a misrepresentation" was clearly [**44] erroneous. That Lin did not recall whether he first screened the monkey cDNA library with a full set of probes or a subset of probes, and his answer that "it looks like" he used the subset, are certainly not clear admissions that he only used a subset. However, the district court was correct in concluding that, even if there had been an erroneous statement, it was not material because Lin succeeded in cloning the EPO gene first with his use of the fully-degenerate probes. Thus, his testimony does not provide clear and convincing evidence that he misrepresented to the PTO the number of probes used. He did use 128-member probes as well as a subset. Moreover, this evidence does not create an inference of an intent to mislead. The court properly concluded that there was no inequitable conduct in prosecuting the *'008* patent.

II. GI's *'195* PATENT (Hewick)

A. *Enablement of claims 1 and 3*

Amgen challenges the district court's determination that "the *'195* patent enables a person of ordinary skill in the art to obtain homogeneous EPO [including rEPO and uEPO] from natural sources" having a mean *in vivo* specific activity of at least 160,000. n10 *13 U.S.P.Q.2d at 1794.* Claims 1 and 3 contain [**45] the limitation that EPO have a specific activity of at least 160,000 [*1216] IU/AU. The district court found, based upon expert testimony from both sides, that to those skilled in the art, in the absence of an express statement in the patent, the claims would be construed to refer to *in vivo* rather than *in vitro* specific activity. To support its challenge, Amgen asserts that the district court's determination is contradicted by GI's own bioassay data and by the district court's finding that "the *'195* patent fails to enable the purification of rEPO." Amgen also asserts that the district court erred in relying solely on an *in vitro* measure of specific activity, having initially construed the *'195* claims as requiring an *in vivo* measure to avoid invalidity for indefiniteness.

n10 The potency of EPO in the *'195* patent is stated as its specific activity, expressed as a ratio of International Units (which measures the ability of EPO to cause formation of red blood cells) per absorbance unit (the amount of light absorbed by a sample of EPO measured by a spectrophotometer at a given wavelength, 280 nanometers), *i.e.*, IU/AU.

[**46]

35 U.S.C. § 112 requires that an invention be described "in such full, clear, concise, and exact terms as to enable any person skilled in the art ... to make and use the same." We review a determination of enablement as a question of law. *Moleculon Research Corp. v. CBS, Inc., 793 F.2d 1261, 1268, 229 U.S.P.Q. (BNA) 805, 811 (Fed. Cir. 1986), cert. denied, 479 U.S. 1030, 93 L. Ed. 2d 829, 107 S. Ct. 875 (1987).*

We do not consider the court's finding that the assay measurement was an *in viva* one to be erroneous in view of the testimony it heard. That being the case, the question is whether the court erred in concluding that the claims requiring 160,000 IU/AU by an *in vivo* measurement were enabled. We conclude that it did err.

Defendants have produced no evidence that it ever prepared EPO with a specific activity of at least 160,000 IU/AU *in vivo* using the disclosed methods. In its report to the FDA, GI stated that it had purified uEPO material "to homogeneity" by subjecting partially purified uEPO material to reverse phase high performance liquid chromatography (RP-HPLC), the technique taught by

Hewick in the *'195* patent. The district court found that GI reported to the FDA that the specific activity of [**47] uEPO, based on *in vivo* bioassays, was only 109,000 IU/AU. n11 GI originally arrived at the figure of 160,000 IU/AU by calculation, before it had the capacity to derive quantitative information from bioassays. Hewick subjected the EPO to RP-HPLC, the EPO having an actual value of 83,000 IU/AU. After weighing the chromatograph, he found that "at least fifty percent" of the area under the chromatograph curve was attributable to something other than EPO. He then doubled the 83,000, and arrived at a theoretical specific activity of "at least about 160,000 IU/AU." That procedure, while possibly valid as a means for estimating the specific activity of a pure sample, does not establish that GI had a workable method for actually obtaining the pure material that it claimed.

n11 Defendants provided no evidence that faulty purification procedures or other missteps caused its failure to obtain 160,000 IU/AU *in vivo* material as claimed in the *'195* patent.

Moreover, the work of others shows that Hewick did not enable the preparation [**48] of uEPO having an *in vivo* specific activity of at least 160,000, as the claims required. Dr. Kawakita, a scientist at Kummamoto University in Japan, reported an *in vivo* specific activity of 101,000 IU/AU when using RP-HPLC according to Hewick's method. This is similar to the 109,000 value reported to the FDA by GI. Kawakita did report a value of 188,000, but did not follow the teachings in the *'195* patent. Defendants also rely on the testimony of Fritsch that "I've also seen further data in Chugai's PLA indicating additional urinary EPO preparation that had activities of 190,000, I believe, units per absorbance unit." However, the document to which Fritsch referred was not offered into evidence by GI after Amgen objected to its introduction and is not before us.

Defendants argue that Dr. Kung's uEPO test result of 173,640 IU/AU in an *in vitro* test supports the enablement of its claims. Amgen argues that an *in vivo* test result would only have been 65 percent of the *in vitro* result and thus would not have met the 160,000 IU/AU limitation of the claims. The district court relied on Kung, despite the demonstrated disparity between the results of *in vitro* and [**49] *in vivo* testing.

It is not absolutely clear to us that, for uEPO, the *in vivo* specific activity is 65 percent of the *in vitro* specific activity. [*1217] Nonetheless, Kung's measurement, being *in vitro*, does not demonstrate enablement of the claimed invention, and that fact means that the court erred in finding enablement. Added to this fact is the difference that exists between the *in vivo* results for rEPO and uEPO, n12 and the other lack of support for the 160,000 limitation. Under these circumstances, we hold that the district court erred in accepting the *in vitro* data as support for claims containing what has been found to be an *in vivo* limitation.

n12 The court quoted Chugai to the effect that the *in vivo* activity of uEPO is 65 percent that of rEPO.

In addition to the question of enablement regarding uEPO, the district court found that the only purification attempt on rEPO in the manner set out in the *'195* patent failed to provide homogeneous EPO. The patent itself, in Example 2, discloses [**50] GI's purification efforts on rEPO and indicates that GI did not obtain purified rEPO. As the district court found, "the patent does not contain any procedures ... for purifying rEPO to the point that RP-HPLC will be successful." *13 U.S.P.Q.2d at 1758.* Thus, the patent fails to enable purification of either rEPO or uEPO. n13 *See In re Rainer, 54 C.C.P.A. 1445, 377 F.2d 1006, 1012, 153 U.S.P.Q. (BNA) 802, 807 (1967)* ("specification is evidence of its own inadequacy").

n13 Chugai's sample reported to the Food and Drug Administration was not purified by the disclosed process.

The burden of showing non-enablement is Amgen's, not GI's, but in the case of a challenged patent, when substantial discovery has occurred, and there is no credible evidence that the claimed purified material can be made by those skilled in the art by the disclosed process, and all evidence from both the inventor and his assignee and from third parties is to the contrary, we conclude that Amgen has met its burden to show that the claims have not been adequately enabled. [**51] We do not hold that one must always prove that a disclosed process operates effectively to produce a claimed product. But, under these circumstances, we conclude that the court erred in holding that claims 1 and 3 were properly enabled.

B. *Indefiniteness of claims 4 and 6*

The district court held claims 4 and 6 of the *'195* patent invalid because their specific activity limitation of

"at least about 160,000" was indefinite. Defendants challenge this holding, asserting that there is no evidence that claims 4 and 6 do not comply with the requirements of *35 U.S.C. § 112.*

The statute requires that "the specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention." A decision as to whether a claim is invalid under this provision requires a determination whether those skilled in the art would understand what is claimed. *See Shatterproof Glass Corp. v. Libbey-Owens Ford Co., 758 F.2d 613, 624, 225 U.S.P.Q. (BNA) 634, 641 (Fed. Cir. 1985)* (Claims must "reasonably apprise those skilled in the art" as to their scope and be "as precise as the subject matter permits."). The district court found [**52] that "bioassays provide an imprecise form of measurement with a range of error" and that use of the term "about" 160,000 IU/AU, coupled with the range of error already inherent in the specific activity limitation, served neither to distinguish the invention over the close prior art (which described preparations of 120,000 IU/AU), nor to permit one to know what specific activity values below 160,000, if any, might constitute infringement. *13 U.S.P.Q.2d at 1787.* It found evidence of ambiguity in the fact that Chugai, GI's partner, itself questioned whether the specific activity value of 138,000 IU/AU for its own rEPO was within the claim coverage.

In prosecuting the *'195* patent, GI disclosed to the examiner a publication by Miyake et al., which discloses a uEPO product having an *in vivo* specific activity of 128,620 IU/AU. When the examiner noticed this disclosure late in the prosecution, he rejected the *'195* claims with a specific activity limitation of "at least 120,000" as anticipated by the Miyake et al. disclosure. [*1218] It was only after the "at least 120,000" claims were cancelled that GI submitted the "at least about 160,000" claim language.

The court found the "addition of the word [**53] 'about' seems to constitute an effort to recapture ... a mean activity somewhere between 120,000, which the patent examiner found was anticipated by the prior art, and [the] 160,000 IU/AU" claims which were previously allowed. Because "the term 'about' 160,000 gives no hint as to which mean value between the Miyake et al. value of 128,620 and the mean specific activity level of 160,000 constitutes infringement," the court held the "at least about" claims to be invalid for indefiniteness. *13 U.S.P.Q.2d at 1787-88.* This holding was further supported by the fact that nothing in the specification, prosecution history, or prior art provides any indication as to what range of specific activity is covered by the term "about," and by the fact that no expert testified as to a definite meaning for the term in the context of the prior art. In his testimony, Fritsch tried to define "about" 160,000, but he could only say that while "somewhere between 155[,000] might fit within that number," he had not "given a lot of direct considerations to that. ..."

When the meaning of claims is in doubt, especially when, as is the case here, there is close prior art, they are properly declared invalid. *Standard* [**54] *Oil Co. v. American Cyanamid Co., 774 F.2d 448, 453, 227 U.S.P.Q. (BNA) 293, 297 (Fed. Cir. 1985).* We therefore affirm the district court's determination on this issue. We also note that, in view of our reversal of the district court's holding that claims 1 and 3 are valid, it is clear that claims 4 and 6 would also be invalid without the "about" limitation. In arriving at this conclusion, we caution that our holding that the term "about" renders indefinite claims 4 and 6 should not be understood as ruling out any and all uses of this term in patent claims. It may be acceptable in appropriate fact situations, *e.g., W.L. Gore & Assocs., Inc. v. Garlock, Inc., 721 F.2d 1540, 1557, 220 U.S.P.Q. (BNA) 303, 316 (Fed. Cir. 1983)* ("use of 'stretching ... at a rate exceeding about 10% per second' in the claims is not indefinite"), even though it is not here.

C. *Inequitable Conduct*

The district court concluded that GI did not engage in inequitable conduct with respect to the *'195* patent. Amgen challenges this holding, asserting, *inter alia*, that GI displayed an intent to mislead by withholding data showing *in vivo* specific activity of homogenous uEPO and withholding information on the range [**55] of error in EPO bioassays.

It is fundamental that to establish inequitable conduct, an intent to deceive is required. *RCA Corp. v. Data General Corp., 887 F.2d 1056, 1065, 12 U.S.P.Q.2d (BNA) 1449, 1456-57 (Fed. Cir. 1989).* A finding of an intent to deceive may follow from an assessment of materiality, knowledge, and surrounding circumstances, including evidence of good faith. *Kingsdown Medical Consultants Ltd. v. Hollister Inc., 863 F.2d 867, 876, 9 U.S.P.Q.2d (BNA) 1384, 1392 (Fed. Cir. 1988), cert. denied, 490 U.S. 1067, 104 L. Ed. 2d 633, 109 S. Ct. 2068 (1989).* The district court found no such intent, stating:

the record is devoid of any evidence that would establish deliberate knowing withholdings of any kind by Dr. Hewick or GI. Dr. Hewick was a credible witness who spoke carefully and candidly about his work ... There is no evidence that Dr. Hewick withheld any information he believed was material to the patent examiner.

Amgen, 13 U.S.P.Q.2d at 1791. There is no clear error in this finding. Amgen raises no inequitable conduct issues that were not fully considered by the district court. We have reviewed the record and find no abuse of discretion on the part of the district court. This is also not an exceptional [**56] case.

III. OTHER ISSUES

In view of our conclusion that the district court erred as a matter of law in holding that claims 1 and 3 of the *'195* patent are not invalid, we vacate the district court's holdings relating to infringement of those [*1219] claims. We have considered the other arguments by counsel on both sides and find them to be without merit.

CONCLUSION

We conclude that the district court did not err in its findings that claims 2, 4, and 6 of the *'008* patent are valid and enforceable and have been infringed by GI, and that claims 7, 8, 23-27, and 29 of the *'008* patent are invalid; we therefore affirm the judgment of the court regarding the *'008* patent. Because we conclude that claims 1, 3, 4, and 6 of the *'195* patent are invalid, we affirm the judgment concerning claims 4 and 6 and reverse the judgment concerning claims 1 and 3.

COSTS

Each party shall bear its own costs.

AFFIRMED-IN-PART, REVERSED-IN-PART, VACATED-IN-PART.

424 f2d 1382

In re David W. Wilson

No. 8271

United States Court of Customs and Patent Appeals

57 C.C.P.A. 1029; 424 F.2d 1382; 1970 CCPA LEXIS 378; 165 U.S.P.Q. (BNA) 494

Oral argument February 4, 1970

May 7, 1970

PRIOR HISTORY:
[***1]

APPEALS from Patent Office, Serial No. 332,321

DISPOSITION:
Reversed.

COUNSEL:
Oberlin, Maky, Donnelly & Renner, William E. Thomson, Jr., John C. Oberlin, attorneys of record, for appellant.

Joseph Schimmel for the Commissioner of Patents. *Raymond E. Martin,* of counsel.

OPINIONBY:
LANE

OPINION:

[**1382]

[*1029] Before RICH, ALMOND, BALDWIN, LANE, Associate Judges, and FORD, Judge, sitting by designation.

LANE, Judge, delivered the opinion of the court.

This appeal is from the decision of the Patent Office Board of Appeals, which affirmed the rejection of claims 1-4, 8-10, and 15-21 in appellant's application serial No. 332,321, filed November 5, 1963, for "Treated Brush and Brush Treating Composition." Four other claims have been allowed. We conclude that the board's decision must be reversed.

The Disclosure

Appellant's disclosure discusses certain problems in the treatment of power-driven rotary brushes. According to the disclosure, it was desirable to produce [**1383] a composition for treating the brush bristles, whereby the ability of the bristles to hold abrasive particles would be enhanced. It discloses that the treatment composition should have a [***2] strength of adhesion to the brush bristles sufficiently great to prevent such composition from transferring excessively to the object being brushed; that the treatment material should wear at substantially the same rate as the brush bristles; that the material should have a high temperature softening point; and that the strength of adhesion between the treating composition and the abrasive particles must be sufficient to withstand the centrifugal force which normally would tend to throw the abrasive outwardly from the brush. The disclosure [*1030] states that previously known brush-treating compositions did not accomplish all these objectives and had a tendency to dry and lose their tackiness over a period of time, thus becoming useless for holding abrasive particles on the bristles.

The disclosure states that appellant discovered that a composition having a high temperature softening point and a high degree of tackiness could be produced if a film-forming resin were blended with a tackifier resin which was incompatible with (insoluble in) the film-forming resin. The resulting composition would have two distinct phases: a continuous phase comprised of film-forming resin, [***3] either alone or saturated with

a small quantity of tackifier resin, and a dispersed phase comprised of small particles of tackifier resin. The two resins may be either completely or partially incompatible, and the disclosure states that the more insoluble the resins, the greater the tack which the composition possesses. Appellant also disclosed that certain plasticizer could be added to render the resins more incompatible, thus further increasing the tack of the composition. Finally, appellant stated that the entire composition could be dissolved in a volatile solvent to allow easy application to the brush, the solvent being one which quickly evaporates upon such application.

The specification contains a list of suitable film-forming resins, including ethyl cellulose, nitro cellulose, cellulose acetate, polyvinyl acetate and cis-polyisoprene, among other materials. A list of tackifiers is given, including certain esters of abietic acid, polyvinyl ethyl ether, coumarone indene resin and terpene resins. A list of plasticizers is also given. The specification then gives four examples showing how to combine various film-formers, tackifiers, plasticizers and solvents to obtain [***4] brush-treating compositions of the desired characteristics, and explains how to apply them to brushes.

The Claims

In view of the result we reach, we find that claims 1 and 8 are representative:

1. A two-phase brush treating composition having a high softening point and sufficient tack to retain abrasive material firmly adhered to brush fill material comprising a film-forming resin and a tackifier resin which is incompatible with said film-forming resin, said two phases comprising a continuous phase formed of said film-forming resin and a dispersed phase formed of small particles of tackifier resin.

8. In conbination, a rotary brush having brush fill material and a two-phase pressure sensitive adhesive brush treating composition adhered thereto having a high softening point and sufficient tack to retain abrasive material firmly adhered to such brush fill material comprising a film-forming resin and a tackifier resin which is incompatible with said film-forming resin, said two phases [*1031] comprising a continuous phase formed of said film-forming resin and a dispersed phase formed of small particles of tackifier resin. [**1384]

The remaining claims on appeal [***5] are narrower, containing recitations of specific resins, plasticizers, etc.

The Prior Art

Grantham n1 relates to coatings for film material and discloses a coating composition comprising a cellulose derivative film-former, a blending resin, a plasticizer, and an organic solvent. Grantham teaches that the blending agent and the film-former should be compatible.

n1 U.S. Pat. *3,051,670*, issued August 28, 1962.

Depew n2 teaches the preparation of emulsions consisting of a continuous phase of water and a discontinuous phase of elastomer particles and particles of a volatile hydrocarbon, with vulcanizing ingredients and other additives dispersed in the hydrocarbon particles. Depew then states that where a dispersion with additional adhesive properties is desired, an adhesive, such as certain of the tackifier resins disclosed by appellants, can be added to the emulsion, and that

n2 U.S. Pat. *2,933,469*, issued April 19, 1960.

[this] adhesive can be water soluble or dispersed as particles. *** The chemistry of the adhesive component is not critical to this invention. The important thing is that the deposited film shall be tacky and adhesive.

Sergi n3 relates [***6] to adhesives suitable for installation of floor-covering products such as linoleum. Sergi's composition consists of a tackifier resin dispersed in a latex binder; the tackifier and latex must be compatible with one another, according to the Sergi disclosure.

n3 U.S. Pat. *3,015,638*, issued January 2, 1962.

Vaughan n4 teaches impregnating a fibrous buffing wheen with an aqueous emulsion consisting of a tacky resin and an emulsifier or stabilizer such as glue or gum.

n4 U.S. Pat *2,890,136*, issued June 9, 1959.

The Board

The board found the composition claims to be unpatentable over Depew, Sergi or Grantham under *35 U.S.C. 103*. The board reached this conclusion after noting that each of the three references shows some of the film-formers, tackifiers, plasticizers and solvents appearing in appellant's lists. The board found that the recited limitation of incompatibility was too relative a term to distinguish over the composition of the references.

The board found that the claims to the treated brush were unpatentable, under *35 U.S.C. 103*, over Vaughan in view of Sergi or Depew. Since Vaughan shows treating brushes, the board apparently considered [*1032] [***7] it obvious to treat brushes with

composition which it thought were made obviously by Sergi or Depew.

The Board also affirmed the rejection of certain claims for being "broader than the disclosure" under *35 U.S.C. 112.* The board's basis for this rejection was that the specification did not provide adequate guidelines for making a selection among the various disclosed ingredients, nor among other materials which are not disclosed but would be included by the claims.

Opinion

We first treat the rejection under section 112. This rejection is in effect an attack on the specification as being insufficient to teach how to practice the broad invention claimed. The rejection is therefore under the first paragraph of section 112. The board's position, as mentioned above, was that the specification did not teach how to select ingredients so that the desired incompatibility would result. We disagree with the board's position on this point. First of all, appellant provided four examples, each specifying the nature and amounts of materials to be used. Secondly, the record indicates that it involves only routine experimentation to find out which resins are incompatible. The examiner admitted [***8] as much when, [**1385] with regard to obviousness, he said "selecting the proper tackifier and film-forming resin from those listed in the references to form an emulsion or two-phase composition would be within the expected skill of the art and would merely involve routine experimentation." We conclude that appellant has provided a sufficient specification to support the claims here in issue.

[1] Turning to the rejection of the claims for obviousness, we again disagree with the board's position. The board has disregarded the term "incompatible," and used in the claims, because it is "too relative" to distinguish over the compositions of the references. Appellant contends this limitation is essential in defining his invention.There has been no rejection here for indefiniteness, under the second paragraph of section 112. Rather than reject the claims as indefinite, the board chose to ignore the language it considered indefinite, and proceeded as though that language were not in the claims. The board said, in effect, that since we do not know what "incompatible" means, and the rest of the claim defines obvious subject matter, there is no basis for concluding unobviousness. [***9] This reasoning is incorrect. All words in a claim must be considered in judging the patentability of that claim against the prior art. If no reasonably definite meaning can be ascribed to certain terms in the claim, the subject matter does not become obvious - the claim becomes indefinite. In the present case, we think the [*1033] term "incompatible" is defined with reasonable definiteness in the specification. While it is true that the word is not perfectly precise, under the circumstances of the present case there appears to be no other way for appellant to describe his discovery. In any event, the ignoring of this term by the board renders it conclusion of obviousness unsupported. None of the references discloses a two-phase composition of incompatible resins or suggests that such a composition would have the properties disclosed by appellant. Grantham and Sergi both expressly teach that the components of their compositions should be compatible. Neither Vaughan nor Depew uses a resin as the continuous phase. While Depew states, as quoted above, that the adhesive material may be dispersed as particles in the continuous phase, and hence be incompatible with the continuous [***10] phase material, it cannot be ignored that Depew's continuous phase is of water, not a film-forming resin as recited in appellant's claims. Furthermore, there is no suggestion in Depew or Vaughan that there are advantages in using an adhesive which in insoluble in the aqueous phase. There is nothing of record, therefore, from which we can properly conclude that the subject matter of appellant's claims would have been obvious at the time of his invention. The decision of the board must accordingly be reversed.

802 f2d 1367

HYBRITECH INCORPORATED, Appellant, v. MONOCLONAL ANTIBODIES, INC., Appellee

Appeal No. 86-531

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

802 F.2d 1367; 1986 U.S. App. LEXIS 20347; 231 U.S.P.Q. (BNA) 81

September 19, 1986

PRIOR HISTORY:
[**1]

Appealed from: U.S. District Court for the Northern District of California. Judge Conti.

DISPOSITION:
Reversed and Remanded.

COUNSEL:
Douglas E. Olson, Lyon & Lyon, of Los Angeles, California, argued for Appellant. With him on brief were James W. Geriak and Bradford J. Duft.

David J. Brezner, Flehr, Hohback, Test, Albritton & Herbert, of San Francisco, California, argued for Appellee. Barry E. Britschneider and Herbert I. Cantor, of Washington, District of Columbia, of Counsel.

JUDGES:
Rich, Davis, and Smith, Circuit Judges.

OPINIONBY:
RICH

OPINION:

[*1368] RICH, Circuit Judge.

This appeal is from the August 28, 1985, decision of the *United States District Court for the Northern District of California, 623 F. Supp. 1344, 227 U.S.P.Q. (BNA) 215,* in favor of defendant Monoclonal Antibodies, Inc. (Monoclonal) holding that all 29 claims of plaintiff's patent No. 4,376,110 entitled "Immunometric Assays Using Monoclonal Antibodies" ('110 patent), issued to Dr. Gary S. David and Howard E. Greene and assigned to Hybritech Incorporated (Hybritech), are invalid as anticipated under *35 USC § 102*(g), for obviousness under § 103, and under § 112 first [**2] and second paragraphs. We reverse and remand.

Background

Vertebrates defend themselves against invasion by microorganisms by producing antibodies, proteins which can complex with the invading microorganisms and target them for destruction or removal. In fact, any foreign molecule of sufficient size can act as a stimulus for antibody production. Such foreign molecules, or antigens, bear particular sites or epitopes that represent antibody recognition sites. B cell lymphocytes, the cells that actually produce antibodies, recognize and respond to an epitope on an antigen by reproducing or cloning themselves and then producing antibodies specific to that epitope. Even if the antigen is highly purified, the lymphocytes will produce antibodies specific to different epitopes on the antigen and so produce antibodies with different specificities. Furthermore, because the body is exposed to many different antigens, the blood of a vertebrate will contain antibodies to many different antigenic substances.

Scientists and clinicians have long employed the ability of antibodies to recognize and complex with antigens as a tool to [*1369] identify or label particular cells or molecules [**3] and to separate them from a mixture. Their source of antibodies has been primarily the serum separated from the blood of a vertebrate immunized or exposed to the antigen. Serum, however, contains a mixture of antibodies directed to numerous antigens and to any number of epitopes on a particular antigen. Because such a mixture of antibodies arises from many different clones of lymphocytes, it is called "polyclonal."

Recent technological advances have made it possible to isolate and cultivate a single clone of lymphocytes to

obtain a virtually unlimited supply of antibodies specific to one particular epitope. These antibodies, known as "monoclonal antibodies" because they arise from a single clone of lymphocytes, are produced by a relatively new technology known as the hybridoma. Hybridomas are produced by fusing a particular cancer cell, the myeloma cell, with spleen cells from a mouse that has been injected or immunized with the antigen. These fusions are isolated by transferring them to a growth fluid that kills off the unfused cancer cells, the unfused spleen cells dying off by themselves. The fused hybrid spleen and myeloma cells, called hybridomas, produce antibodies to the [**4] antigen initially injected into the mouse. The growth fluid containing the hybridomas is then diluted and put into individual test tubes or wells so that there is only one hybridoma per tube or well. Each hybridoma then reproduces itself and these identical hybridomas each produce identical monoclonal antibodies having the same affinity and specificity. In this way, a virtually unlimited supply of identical antibodies is created, directed to only one epitope on an antigen rather than, as with polyclonal antibodies, to many different epitopes on many different antigens.

In addition to the specificity of antibodies to particular epitopes discussed above, antibodies also have a characteristic "sensitivity," the ability to detect and react to antigens. Sensitivity is expressed in terms of "affinity:" the greater an antibody's ability to bind with a particular antigen, the greater the antibody's affinity. The strength of that antibody-antigen bond is in part dependent upon the antibody's "affinity constant," expressed in liters per mole, for the antigen.

Immunoassays, the subject matter of the '110 patent, are diagnostic methods for determining the presence or amount of antigen in [**5] body fluids such as blood or urine by employing the ability of an antibody to recognize and bind to an antigen. Generally, the extent to which the antibody binds to the antigen to be quantitated is an indication of the amount of antigen present in the fluid. Labelling the antibody or, in some cases, the antigen, with either a radioactive substance, I125, or an enzyme makes possible the detection of the antibody-antigen complex. In an extreme case, where the fluid sample contains a very low level of the antigen, binding might not occur unless the antibodies selected or "screened" for the procedure are highly sensitive.

In the case of a "competitive" immunoassay, a labelled antigen reagent is bound to a limited and known quantity of antibody reagent. After that reaction reaches equilibrium, the antigen to be detected is added to the mixture and competes with the labelled antigen for the limited number of antibody binding sites. The amount of labelled antigen reagent displaced, if any, in this second reaction indicates the quantity of the antigen to be detected present in the fluid sample. All of the antigen attached to the antibody will be labelled antigen if there is no antigen [**6] in the test fluid sample. The advantage of this method is that only a small amount of antibody is needed, its drawback, generally, that the system must reach equilibrium, and thus produces results slowly.

In the case of a "sandwich" assay, otherwise known as an immunometric assay, the latter being a term coined by Dr. Lawton Miles in 1971, a quantity of unlabelled antibody reagent is bound to a solid support surface such as the inside wall of a test tube containing a complex of the fluid sample [*1370] containing the antigen to be detected and a labelled *antibody* reagent. The result is an insoluble three part complex referred to as a sandwich having antibody bread and antigen filling. This figure is illustrative of the sandwich concept:

[SEE ILLUSTRATION IN ORIGINAL]

The advantage of the sandwich assay is that it is fast and simple, its drawback that enormous quantities of antibodies are needed.

Hybritech

Hybritech, started in 1978 and joined thereafter by coinventors Green and Dr. David, has, since 1979, been in the business of developing diagnostic kits employing monoclonal antibodies that detect numerous antigens and thus a broad range of conditions [**7] such as pregnancy, cancer, growth hormone deficiency, or hepatitis. Examples of antigens include influenza viruses, immunoglobulin E (IgE) which indicates allergic reaction, human chorionic gonadotropin (HCG) which indicates pregnancy, and prostatic acid phosphatase (PAP) which indicates prostate cancer, to name a few. Dr. Adams, a business-experienced scientist, joined the company in May 1980 as head of research and development. The '110 patent, application for which was filed August 4, 1980, issued March 8, 1983, with claims defining a variety of sandwich assays using monoclonal antibodies. Claim 19, apparently the broadest of the twenty-nine in the patent, is directed generally to a sandwich assay and reads (emphasis ours):

19. *In an immunometric assay* to determine the presence or concentration of an antigenic substance in a sample of a fluid comprising forming a ternary complex of a first labelled antibody, said antigenic substance, and a second antibody said second antibody being bound to a solid carrier insoluble in said fluid wherein the presence of the antigenic substance in the samples is determined by measuring either the amount of labelled antibody bound to [**8] the solid carrier or the amount of unreacted labelled antibody, *the improvement comprising*

employing monoclonal antibodies having an affinity for the antigenic substance of at least about 10<8> liters/mole for each of said labelled antibody and said antibody bound to a sold carrier.

Claim 1, directed particularly to a reverse sandwich assay, explained infra, reads:

1. A process for the determination of the presence of [sic, or] concentration of an antigenic substance in a fluid comprising the steps:

(a) contacting a sample of the fluid with a measured amount of a soluble first monoclonal antibody to the antigenic substance in order to form a soluble complex of the antibody and antigenic substance present in said sample, said first monoclonal antibody being labelled;
(b) contacting the soluble complex with a second monoclonal antibody to the antigenic substance, said second monoclonal antibody being bound to a solid carrier, said solid carrier being [*1371] insoluble in said fluid, in order to form an insoluble complex of said first monoclonal antibody, said antigenic substance and said second monoclonal antibody bound to said solid carrier;
(c) [**9] separating said solid carrier from the fluid sample and unreacted labelled antibody;
(d) measuring either the amount of labelled antibody associated with the solid carrier or the amount of unreacted labelled antibody; and
(e) relating the amount of labelled antibody measured with the amount of labelled antibody measured for a control sample prepared in accordance with steps (a)-(d), said control sample being known to be free of said antigenic substance, to determine the presence of antigenic substance in said fluid sample, or relating the amount of labelled antibody measured with the amount of labelled antibody measured for samples containing known amounts of antigenic substance prepared in accordance with steps (a)-(d) to determine the concentration of antigenic substance in said fluid sample, the first and second monoclonal antibodies having an affinity for the antigenic substance of at least about 10 <8> liters/mole.

The District Court Decision

Hybritech sued Monoclonal March 2, 1984, for damages and an injunction alleging that the manufacture and sale of Monoclonal's diagnostic kits infringed the '110 patent. Trial without a jury began on August 5, 1985, and [**10] concluded August 23, 1985, thirty witnesses having been heard and over 2,000 pages of transcript generated. The district court produced the reported opinion, findings, and conclusions, which use nearly verbatim Monoclonal's *pre-trial* brief and *pre-trial proposed* findings of fact and conclusions of law, in three days, in support of the judgement now on appeal.

The district court held that the claimed subject matter of the '110 patent was neither conceived nor actually reduced to practice before May 1980, and was anticipated under § 102(g) by the actual reduction to practice of the invention by Drs. Uotila and Ruoslahti at the La Jolla Cancer Research Foundation (LJCRF) as early as November of 1979 and by the actual reduction to practice of the invention by Drs. Oi and Herzenberg (Oi/Herzenberg work) at the Stanford University Laboratory as early as July 1978, later published in December of 1979.

The district court also held the claims of the '110 patent invalid for obviousness from the Oi/Herzenberg work in view of (1) a February 1979 article by M. E. Frankel and W. Gerhard (Frankel article) which discloses high-affinity monoclonal antibodies, and apparently in view of [**11] numerous other references including; (2) the work of Nobel Prize winners G. Kohler and C. Milstein disclosing a Nobel Prize-worthy method for producing monoclonal antibodies in vitro (outside the body) published in an August 7, 1975, article; (3) U.S. Patent No. *4,244,940* issued to Jeong et al. disclosing a simultaneous polyclonal assay (Jeong), U.S. Patent No. *4,098,876* to Piasio et al. disclosing a reverse polyclonal sandwich assay (Piasio), U.S. Patent No. *4,016,143* to Schurrs et al. disclosing a forward polyclonal sandwich assay (Schurrs); (4) a July 1979 publication by A. C. Cuello et al. disclosing the use of monoclonal antibodies in competitive assays; and (5) eight articles dated between January 1979 and March 6, 1980, "predicting" that monoclonal antibodies would be used in future immunoassays. n1

n1 With respect to obviousness, one portion of the district court's opinion apparently relies on all of the above listed references, (1) -- (5), for the obviousness holding while a later portion entitled "CONCLUSIONS OF LAW" relies on only the Oi/Herzenberg and Frankel articles. Furthermore, the district court did not state that the LJCRF work was considered for purposes of § 103, although we recognize that § 102(g) prior art can be used for § 103.

[**12]

The district court also invalidated the patent on various grounds based on *35 USC § 112,* first and second paragraphs, as hereinafter discussed.

[*1372] A. *The References*

1. *Kohler and Milstein's Nobel Prize-Winning Work: Producing Monoclonal Antibodies In Vitro For the First Time*

In early immunoassay work, polyclonal antibodies produced in vivo (in the body) in mice were used to bind with the antigen to be detected in the body fluid sample. Mice were immunized by injection with antigen so that the lymphocytes in their bodies produced antibodies that attacked the injected antigen. Those polyclonal antibodies were withdrawn from the animal's blood and used in immunoassays. The major problem was that when the mice's immune systems changed or the mice died, the antibodies changed or died too; supply was limited and uncertain.

As the examiner was aware, Kohler and Milstein developed a technique not only for producing antibodies in vitro, independent of a living body, thus eliminating dependence on a particular animal, but for in vitro production of monoclonal antibodies by hybridomas, discussed in the Background section, supra.

Given that [**13] sandwich assays require enormous amounts of antibodies, companies like appellant and appellee, which utilize monoclonal antibodies for sandwich assays, would not be in business were it not for the work of Kohler and Milstein.

2. *The Work of Drs. Ruoslahti, Uotila, and Engvall at the La Jolla Cancer Research Foundation (LJCRF) in 1979 and 1980*

Dr. Ruoslahti performed mostly competitive immunoassays using polyclonal antibodies to alphafetoprotein (AFP) antigens at the City of Hope since 1970. Dr. Uotila joined him in late 1978 to perform immunoassays using monoclonal antibodies to AFP. After producing monoclonal antibodies to AFP and performing competitive radio immunoassays (RIA -- a competitive assay that uses a radioactive label) with monoclonal antibodies at the City of Hope in mid-1979, Drs. Ruoslahti, Uotila and Engvall left LJCRF.

In the fall of 1979, September or October according to Dr. Uotila, discussion and work began on using monoclonal antibodies to AFP in a sandwich assay. Dr. Uotila, the principal researcher in this particular endeavor, generated six notebooks while at the City of Hope and LJCRF. The next-to-last page of notebook four contained a note to [**14] Dr. Uotila from Dr. Ruoslahti reading:

Sometime you should enzyme label a good monoclonal antibody so that you can set up a sandwich assay. If you use two monoclonal antibodies, you may be able to do the assay with a single incubation, since the monoclonal antibodies are likely to be directed against different determinants and not compete with one another.

Although Dr. Uotila's notebook pages were, for the most part, unsigned, undated, and uncorroborated, Dr. Ruoslahti's testimony, placed the date of this note at about October 1979 by referring to the first pages of notebook five which were dated in early November 1979. Dr. Ruoslahti testified that one curve on one graph on page 43D of notebook five showed a successful simultaneous sandwich assay using monoclonal antibodies about November 5, 1979, although no data supporting that graph could be found elsewhere in the notebook. He further testified that the affinity of the monoclonal antibodies used for that test was not calculated until 1980 but that the raw data necessary for that calculation was generated in 1979.

Dr. Uotila stated in her deposition (she did not testify at trial) that she started work on a sandwich [**15] assay using monoclonal antibodies between October 4 and the end of that month, 1979, and that she could not remember the procedure used nor was there enough information in her notebook, including page 43D, to refresh her memory. She did remember, although she continued work on this assay because the tests did not yield repeatedly good curves without which she would not publish her work, that the assay on page 43D was successful. Dr. Engvall testified about a discussion of Dr. Uotila's monoclonal antibody work with [*1373] her while at the City of Hope and about first performing a sandwich assay after arriving at LJCRF in 1979.

3. *The Work of Drs. Oi and Herzenberg at the Stanford University Laboratory in 1978 Published in December 1979*

Drs. Oi and Herzenberg used monoclonal antibodies to "map" epitopes or determine the number and location of different antibody binding sites on a known quantity of IgE antigen by attaching to it an antibody bound to a carrier and exposing that antigen to other monoclonal antibodies. The antibodies either attached to epitopes on the antigen or were blocked from doing so by the other monoclonal antibodies, depending on the location and [**16] number of epitopes; if the epitopes on the antigen were too close together and the number of antibodies too great, few antibodies would bind to the antigen. Hybritech points out that both Dr. Herzenberg and Dr. Oi testified that *their work did not involve determining the presence or quantity of antigen*, that they had no idea what the affinities of the monoclonal antibodies used were, and that those values were never calculated.

One unsigned, unwitnessed page from three large laboratory notebooks, which Hybritech argues is insufficient because it does not identify the chemical reagents or protocol used, was relied on by Monoclonal to establish actual reduction to practice of the Oi/Herzenberg work in 1978 to establish a case of § 102(g) prior invention by another. The district court agreed with Monoclonal that the Oi/Herzenberg work anticipated the claimed invention and, in addition, combined this work with the Frankel publication to hold that the claimed subject matter was obvious under § 103.

4. *The Frankel Article: Monoclonal Antibodies Having Affinities of 10 (9) liters/mole*

Frankel describes an RIA (radioimmunoassay) method for the rapid determination of affinity [**17] constants for monoclonal antibodies produced from hybridomas. The article states that the assay used is applicable only to antibodies with binding constants of about 10 <10> liters/mole and discloses the binding constants for antibodies to several closely related strains of influenza virus.

The district court found that Frankel disclosed monoclonal antibodies having the affinity constants claimed in the '110 patent, 10 <8> to over 10 <9> liters/mole.

5. *The Cuello Article and the Jeong, Piasio, and Schurr Patents Considered by the Examiner*

Cuello, dated July 1979, states that it describes the usefulness of monoclonal antibodies in the characterization and localization of neurotransmitters such as Substance P, a peptide clearly associated with the transmission of primary sensory information in the spinal cord. The article discloses producing monoclonal antibodies from hybrid myelomas (hybridomas), their use in conventional radioimmunoassay techniques, and the benefits from doing so which flow from the ability to derive permanent cell lines capable of continuous production of highly specific antibodies.

The district court found that the examiner twice rejected all of the [**18] claims of the '110 patent based on Cuello alone or in combination with the Jeong, Piasio, and Schurr references which disclose various sandwich assays using polyclonal antibodies. The court also found that the examiner allowed the claims after they were amended to include the 10 <8> affinity limitation and after Richard Bartholomew, a Hybritech employee, submitted an affidavit alleging the advantages of using monoclonal rather than polyclonal antibodies in sandwich assays.

Apparently based on the testimony of Monoclonal's expert witness Judith Blakemore, a named inventor of the Jeong patent, manager of antibody programs at Bio-Rad Laboratories from 1975 to 1982, and currently manager of monoclonal antibody therapeutics at Cetus Corporation, a Hybritech competitor in immunoassay diagnostics, the district court stated that the "reasons for allowance were not well-founded because (1) the alleged advantages were [*1374] expected as naturally flowing from the well-known natural characteristics of monoclonal antibodies ...; (2) ... were not significant ...; or (3) were at best minor," although they were "argued to the examiner as if they were" important. These were Monoclonal's [**19] words from its pretrial submission adopted by the court.

6. *The References That "Predicted" the Use of Monoclonal Antibodies in Immunoassays*

The district court stated, again in Monoclonal's words, that "it is of the utmost importance" that the advantages of monoclonal antibodies were "predicted by a number of authorities," eight to be exact, not important enough to list here, after the Kohler and Milstein discovery and after monoclonal antibodies became available.

B. *The Claimed Subject Matter of the '110 Patent*

Hybritech argues that the district court's determination that there is no credible evidence of conception or reduction to practice of the '110 invention before May 1980 is error because Dr. David's laboratory notebooks, Nos. 21 and 24, clearly show successful sandwich assays using monoclonal antibodies in August, September, and October of 1979. At the least, argues Hybritech, the invention was conceived in January of 1979, long before Drs. Ruoslahti, Engvall, and Uotila began work on a sandwich assay using monoclonal antibodies, and diligence was thereafter exercised until constructive reduction to practice occurred by the filing of the '110 patent application [**20] on August 4, 1980.

Dr. David and Greene testified that pages 2118 to 2122 of Dr. David's notebook, dated January 4, 1979, and witnessed January 30, 1979, disclose the generic conception of the invention in the context of the physical support structure used to carry out a sandwich assay, and Dr. David testified on redirect that (1) Page 1128 of notebook 21, dated May 27, 1979, recorded an early attempt at a sandwich assay that failed, (2) on August 3, 1979, as recorded at page 1166, a sandwich assay using monoclonal antibody 068 attached to a solid carrier, a radio-labelled 068 antibody, and a hepatitis antigen from an Abbott Labs polyclonal competitive assay kit was successfully performed, and (3) a sandwich assay using a bound 259 antibody, a radio-labelled 068 antibody, and a hepatitis antigen was successfully performed on September 21, 1979. Hybritech also urges that work in October 1979 directed to determining whether certain monoclonal antibodies were recognizing the same or different determinants, was a reduction to practice.

Monoclonal points out that these notebook pages do not expressly state that monoclonal antibodies of 10 <8> liters/mole affinity were used in a sandwich [**21] assay and that the May, August, and September notebook entries were not witnessed until about the time Dr. Adams, experienced in patent matters, joined Hybritech and advised its researchers on properly recording laboratory work. They therefore claim that actual reduction to practice was not shown before May 1980.

OPINION

I. *Review Under Rule 52(a) Fed. R. Civ. P.*

Rule 52(a) "ensures care in the preparation of an opinion ... and provides appellate courts with the benefit of the District Court's insights into a case," *Pentec, Inc. v. Graphic Controls Corp., 776 F.2d 309, 318, 227 U.S.P.Q. (BNA) 766, 772 (Fed. Cir. 1985)* (Harvey, Senior District Judge, concurring) by requiring a district court to "find the facts specially and state separately its conclusions of law thereon." With the exception of the first eight paragraphs, the first half of the district court's opinion here is Monoclonal's *pretrial* brief and the last three pages of the opinion are Monoclonal's *pretrial* findings of fact and conclusions of law. The district court adopted the above documents [**22] virtually verbatim, with the exception of portions of each concerning inequitable conduct and noninfringement, apparently without inviting a response from Hybritech, resulting in a repetitious (as the district court admitted in [*1375] the opinion), sometimes internally inconsistent, and hard to follow opinion that presents us with a difficult task in gleaning the basis for many of the conclusions. For some of the findings, submitted before trial, no supporting evidence was introduced at trial.

The Supreme Court, in *Anderson v. City of Bessemer City, N.C., 470 U.S. 564, 105 S. Ct. 1504, 84 L. Ed. 2d 518 (1985),* strongly criticized the practice of "verbatim adoption of findings of fact prepared by prevailing parties, particularly when those findings have taken the form of conclusory statements unsupported by citation to the record." *Anderson*, supra at 1511. This court also has cautioned against the adoption of findings, especially when proposed by a party before trial, as here, and stated that the likelihood of clear error in those findings increases in such a situation. *Lindemann Maschinenfabrik v. American Hoist and Derrick, 730 F.2d 1452, 221 U.S.P.Q. (BNA) 481, 485 (Fed. Cir. 1984).* [**23] Notwithstanding our misgivings about whether the findings in this case, prepared before any evidence was introduced, satisfy the objectives of Rule 52(a) -- a carefully prepared opinion providing the reviewing court with the benefit of the district court's *reasoned insights* into the case -- those findings are the district court's and may be reversed only if clearly erroneous. *See Anderson*, supra, at 1511; *Lindemann, 730 F.2d at 1457, 221 U.S.P.Q. at 485.*

"A finding is clearly erroneous when, although there is evidence to support it, the reviewing court on the entire evidence is left with the definite and firm conviction that a mistake has been committed." *United States v. United States Gypsum Co., 333 U.S. 364, 395, 92 L. Ed. 746, 68 S. Ct. 525 (1948).* "This standard plainly does not entitle a reviewing court to reverse the finding of the trier of fact simply because it is convinced that it would have decided the case differently." *Anderson*, supra, at 1511. In other words, "if the district court's account of the evidence is plausible in light [**24] of the record viewed in its entirety" or "where there are two permissible views of the evidence," the factfinder cannot be clearly erroneous. *Anderson*, supra, at 1511 (quoting *United States v. Yellow Cab Co., 338 U.S. 338, 70 S. Ct. 177, 94 L. Ed. 150 (1949)).* This is so, stated the Court in dictum, *see Anderson*, supra, at 1516 (Blackmun, J., concurring), even when the district court's findings rest on physical or documentary evidence or inferences from other facts and not on credibility determinations. *See also* Rule 52(a) Fed. R. Civ. P. (as amended Aug. 1, 1985). If the latter are involved, "Rule 52 demands even greater deference to the trial court's findings" but a trial judge may not "insulate his findings from review by denominating them credibility determinations"; if documents or objective evidence contradict the witness's story, clear error may be found even in a finding purportedly based on a credibility determination. *Anderson*, supra, at 1512-13. We proceed in light of all these principles.

II. *Presumption of Validity*

[**25] Under *35 USC § 282,* a patent is presumed valid, and the one attacking validity has the burden of proving invalidity by clear and convincing evidence. *See, e.g., American Hoist & Derrick Co. v. Sowa & Sons, Inc., 725 F.2d 1350, 1360, 220 U.S.P.Q. (BNA) 763, 770 (Fed.Cir. 1984).* Notwithstanding that the introduction of prior art not before the examiner may facilitate the challenger's meeting the burden of proof on invalidity, the presumption remains intact and on the challenger throughout the litigation, and the clear and convincing standard does not change. *See, e.g., Jervis B. Webb Co. v. Southern Systems, Inc., 742 F.2d 1388, 1392 & n.4, 222 U.S.P.Q. (BNA) 943, 945 & n.4 (Fed. Cir. 1984).* The only indication that the district court recognized the presumption of validity and its proper application was its statement that "the key issue in this case is whether the defendant has overcome the presumption of nonobviousness." That statement, however, speaks only part of the truth; the presumption of validity goes to validity of the patent in relation to the patent statute *as a*

[**26] *whole*, not just to nonobviousness under section 103.

[*1376] III. *Prior Invention of Another, 35 USC § 102(g)*

Section 102(g) states that a person shall be entitled to a patent unless "before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it." Section 102(g) "relates to prior inventorship by another in this country" and "retains the rules governing the determination of priority of invention. ..." *Kimberly-Clark Corp. v. Johnson & Johnson, 745 F.2d 1437, 1444, 223 U.S.P.Q. (BNA) 603, 606 (Fed. Cir. 1984)* (quoting P.J. Federico, *Commentary on the New Patent Act, 35 USCA page 1,* at 19 (1954)). Section 102(g) says: "In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other."

[**27] Reduction to practice, and conception as well, is a legal determination subject to review free of the clearly erroneous standard. *Barmag Barmer Maschinenfabrik AG v. Murata Machinery, Ltd., 731 F.2d 831, 837, 221 U.S.P.Q. (BNA) 561, 565-66 (Fed. Cir. 1984); D.L. Auld Co. v. Chroma Graphics Corp., 714 F.2d 1144, 1151, 219 U.S.P.Q. (BNA) 13, 18 (Fed. Cir. 1983).* Findings of fact supporting that legal conclusion are, of course, reviewed under the clearly erroneous standard.

Conception is the "formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice." 1 *Robinson On Patents* 532 (1890); *Coleman v. Dines, 754 F.2d 353, 359, 224 U.S.P.Q. (BNA) 857, 862 (Fed. Cir. 1985).* Actual reduction to practice requires that the claimed invention work for its intended purpose, *see, e.g., Great Northern Corp. v. Davis Core & Pad Co., 782 F.2d 159, 165, 228 U.S.P.Q. (BNA) 356, 358,* (Fed. [**28] Cir. 1986), and, as has long been the law, constructive reduction to practice occurs when a patent application on the claimed invention is filed. *Weil v. Fritz, 572 F.2d 856, 865 n.16, 196 U.S.P.Q. (BNA) 600, 608 n.16 (CCPA 1978)* (citing with approval *Automatic Weighing Machine Co. v. Pneumatic Scale Corp., 166 F. 288 (1st Cir. 1909)).*

After a review of the record in its entirety, including the numerous corroborating Hybritech laboratory notebooks, internal documents, and pertinent testimony, we hold clearly erroneous the district court's finding that there is no clear or corroborated evidence "with regard to when before May 1980, the idea of actually using monoclonals in sandwich assays" was conceived or, more properly, or when the *claimed invention* was conceived, and therefore reverse the court's holding, as a matter of law, that Hybritech's inventors did not conceive the claimed invention before May 1980.

Hybritech's claim of conception, generally, is evidenced by the sometimes sparsely documented work of a start-up company whose first small advances evolved into the myriad activities of a mature company with efforts directed toward developing [**29] the claimed invention by first employing the Kohler and Milstein technology to produce the necessary monoclonal antibodies and using those antibodies in diagnostic sandwich assay kits. There is no doubt that exploiting monoclonal antibodies for use in sandwich assays was one of the major objectives of Hybritech. In a letter to Pharmacia Fine Chemicals dated April 26, 1979, Greene, in responding to Pharmacia's interest in Hybritech's products, outlined the latter's "efforts to bring the exciting new hybridoma technology into routine medical use" and its exploration of "several intriguing concepts for which monoclonals may open up new immunodiagnostic techniques heretofore infeasible with animal serums." Although company minutes in early 1979 contain little about the claimed subject matter and some of the discussions thereon, such as Greene's and Dr. Adams' conversation about monoclonal sandwich assays when the former was trying to woo Dr. Adams to join Hybritech were unrecorded, the Hybritech laboratory notebooks and the [*1377] nature of Hybritech's research program fully corroborate the testimonial evidence of conception and thus clearly support our holding that Hybritech conceived [**30] the claimed invention before LJCRF.

Dr. David's January 1979 notebook describes, in detail, as explained by Greene and Dr. David at trial, a nylon apparatus that undoubtedly could be used for performing a sandwich assay using monoclonal antibodies, although Dr. David testified on cross-examination that at that time Hybritech had not yet developed any monoclonal antibodies, including attaching one of the reagents to a solid carrier ring, contacting that ring with a fluid sample in a microtiter plate well, adding a labelled reagent to the well after rinsing, and then "counting" or measuring the amount of either the labelled or unlabelled reagent after a prescribed time and second rinsing. The notebook then describes the procedure for detecting an antibody "(a-x)" to an antigen "(x)" complete with diagrams and text, both illuminated by Dr. David at trial. The notebook further states, "Alternatively, if one wished to quantitate an antigen, y, the identical procedure would be followed, except that reagents would be reversed, i.e. the reaction would be:" and there follows a clear illustration of an

antibody attached to a solid carrier reacting with an antigen to form a complex, and that [**31] complex reacting with a second labelled antibody. The notebook was signed by Dr. David on January 4, 1979, and witnessed and signed on January 30 of the same year by Dr. Curry, the first cell biologist hired at Hybritech to set up the hybridoma production program.

Dr. David testified on direct that monoclonal antibodies were developed in the following months: antigens were purchased from outside sources and purified before being injected into mice; the spleen cells from those mice were fused with myelomas; and the resultant hybridomas were separated into well plates for development, and a radioimmunoassay procedure was carried out to determine the affinity of the antibodies.

The May 1979 failed sandwich assay, witnessed in May 1980, corroborates Dr. David's testimony that a polyclonal antibody bound to be a solid carrier and a labelled monoclonal antibody were used in a sandwich assay with an antigen from Abbott Labs' Ausria polyclonal diagnostic kit for hepatitis. No binding was detected.

Dr. David testified about the experiment documented in the August 1979 notebook, a sandwich assay with a hepatitis antigen from an Abbott Labs Ausria kit with two Hybritech 068 monoclonal [**32] antibodies, one attached to a solid carrier bead and the other labelled; the purpose of the experiment was to quantitate the antigen. The notebook corroborates Dr. David's testimony that the test was positive and lists the counts per minute of the labelled antibody. Defendant Monoclonal's expert Ciotti testified about this experiment:

Also, of course, it is limited to -- it is limited to hepatitis antigen. And without a generic conception, it would just be merely a -- if it did work for its intended purpose -- which I would assume for purposes of discussion -- *it would be a reduction to practice of one embodiment.* And without a corresponding generic conception, I don't think it would be held to be the making of the invention in terms of, for instance, in claim 19. [Emphasis ours.]

Dr. David further testified that the September 21, 1979, record in David's notebook, witnessed months later, shows a reverse sandwich assay using a bound 259 monoclonal antibody and a labelled 068 monoclonal antibody with a hepatitis antigen with results confirmed by a dose response curve. n2 Hybritech further alleges that a laboratory notebook page dated October 1979 is a reduction [**33] to practice of the [*1378] claimed invention but fails to cite any related testimony or other evidence in support thereof.

n2 A dose response curve is antigen concentration plotted against the signal produced by labelled antibody in an immunoassay. The signal increases with increasing antigen concentration in a successful assay but at some point decreases when the antigen concentration becomes too high.

Finally, the record shows that the claimed affinity limitation "of at least about 10 <8> liters/mole" was determined and appreciated during the course of the development of the claimed subject matter. Dr. David and Dr. Adams separately testified that the screening procedures used by Hybritech ensured that only monoclonal antibodies having at least 10 <8> liters/mole affinity would be used in assays. An October 1979 internal memorandum from Greene to the staff states, "To improve comparisons we will express all affinities to the base ten to the eighth which represents the lower end of the usable range." [**34]

We are left with the definite and firm conviction that a mistake has been committed because the district court's account of the evidence that "there was no credible evidence of conception before May 1980" is insupportable. There is such evidence. The laboratory notebooks, alone, are enough to show clear error in the findings that underlie the holding that the invention was not conceived before May 1980. That some of the notebooks were not witnessed until a few months to one year after their writing does not make them incredible or necessarily of little corroborative value. Admittedly, Hybritech was a young, growing company in 1979 that failed to have witnesses sign the inventors' notebooks contemporaneously with their writing. Under a reasoned analysis and evaluation of all pertinent evidence, however, we cannot ignore that Hybritech, within a reasonable time thereafter, prudently had researchers other than those who performed the particular experiments witness the notebooks in response to Tom Adams' advice. The notebooks clearly show facts underlying and contemporaneous with conception of the claimed invention and in conjunction with the testimony of Dr. David and Greene, [**35] and others, are altogether legally adequate documentary evidence, under the law pertaining to conception, of the formation in the minds of the inventors of a definite and permanent idea of the complete and operative invention as it was thereafter applied in practice. We thus are not moved by Monoclonal's argument that the findings of fact

underlying conception are based on credibility determinations and are more sacrosanct than usual. *See Anderson*, supra, at 1512-13.

1. *LJCRF Is Not Prior Art*

Hybritech laboratory notebooks and the uncontradicted testimony of Dr. David and Mr. Greene show that development of the claimed invention proceeded diligently through the rest of 1979 and 1980, there being absolutely no evidence of record nor even argument by Monoclonal that Hybritech was not diligent in its efforts to reduce to practice the claimed invention during the period January 1979 to the '110 application filing date of August 4, 1980. We therefore hold as a matter of law that Hybritech's conception, which was before LJCRF conceived the claimed invention, coupled by diligence to its constructive reduction to practice by the filing of the '110 application, entitle Hybritech [**36] to priority over LJCRF. *See 35 USC § 102*(g). The work of LJCRF is therefore not prior art.

We also note that there is inadequate factual basis for the district court's holding that LJCRF reduced the claimed invention to practice as early as November 1979 because the only evidence that corroborates the testimony of Ruoslahti, Uotila, and Engvall is the note from Ruoslahti to Uotila, see section A, 2, supra, which indisputably is not the claimed invention, and the *one* curve from *one* graph from only one page, 43D, of the six Uotila notebooks. After a reasoned examination, analysis, and evaluation of this pertinent evidence we conclude that it falls far short of showing the "formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is hereafter to be applied in practice," *see Coleman, 754 F.2d at 359, 224 U.S.P.Q. at 862,* and therefore is legally inadequate to support even a holding of *conception* of [*1379] the claimed invention by LJCRF personnel in 1979.

(1) It is undisputed that page 43D was not signed, witnessed, or dated; (2) the deposition testimony of [**37] Uotila was that she could not remember the procedure used to arrive at the dose-response curve on page 43D and there was not enough information in her notebook to refresh her memory; (3) the testimony of Ruoslahti was that he could find *no* data in the notebook supporting that graph, none of the *later* graphs shown there represented successful assays and that "especially after this was done, we ran into more severe problems. And it took us a while to do away with the problems;" (4) Ruoslahti also testified that they never determined, in 1979, the affinities of the monoclonal antibodies they used, and that the title of page 43D had been altered at some point -- the word "inhibition" had been crossed out and "sandwich" written in; and (5) the testimony of Engvall was that there was nothing about the shape of those curves which indicates that they were sandwich assays. We also note, as evidence bearing upon the credibility of Ruoslahti's testimony (that LJCRF actually reduced the claimed invention to practice in 1979), that when LJCRF attempted to provoke an interference in the PTO with Hybritech based on the U.S. filing of an application that was the counterpart to a Swedish application [**38] disclosing similar subject matter, LJCRF could not demonstrate even a *prima facie* reduction to practice prior to Hybritech's August 4, 1980, filing date. During that proceeding, the earliest dates Ruoslahti set down on paper to support conception and reduction to practice were in 1980.

2. *The Work of Oi/Herzenberg Is Not the Claimed Invention*

It is axiomatic that for prior art to anticipate under § 102 it has to meet every element of the claimed invention, and that such a determination is one of fact. *See, e.g., Lindemann, supra, 730 F.2d at 1458, 221 U.S.P.Q. at 485; Great Northern Corp. v. Davis Core & Pad Co., 782 F.2d 159, 165, 228 U.S.P.Q. (BNA) 356, 358 (Fed. Cir. 1986).* Section 102(g) upon which the district court relied is one type of "anticipation," i.e., prior invention by another of the same invention. Drs. Oi and Herzenberg testified that their work did not involve detecting the presence of or quantitating antigen but a determination of the number and location of epitopes on a *known* quantity of antigen. Although this work did [**39] involve a sandwich assay to the extent that an antigen was sandwiched between two monoclonal antibodies, it is clear that the similarity between that work and the claimed invention goes no further. Furthermore, both doctors testified that they did not know the affinities of the antibodies that were used in their mapping work and in fact never calculated them. Ciotti, Monoclonal's expert, testified that the 10<8> affinity limitation cannot be found anywhere in the Oi/Herzenberg work. Again we are left with a definite and firm conviction that a mistake was made because that work does not meet every element of the claimed invention. The district court's finding to the contrary is clearly erroneous.

We note that the district court, in also holding the patent invalid under § 103, next considered, combined the Oi/Herzenberg work with the Frankel reference, one justifiable inference therefrom being that the court recognized that Frankel discloses a claim *element* that Oi/Herzenberg does not, namely, at least about 10<8> liters/mole affinity.

IV. *Obviousness, 35 USC § 103*

[**40] A section 103 obviousness determination -- whether the claimed invention *would have been* (not

"would be" as the court repeatedly stated because Monoclonal's pretrial papers used that improper language) obvious at the time the invention was made is reviewed free of the clearly erroneous standard although the underlying factual inquiries -- scope and content of the prior art, level of ordinary skill in the art, n3 and differences between the prior art [*1380] and the claimed invention -- integral parts of the subjective determination involved in § 103, are reviewed under that standard. Objective evidence such as commercial success, failure of others, long-felt need, and unexpected results must be considered *before* a conclusion on obviousness is reached and is not merely "icing on the cake," as the district court stated at trial. *See Lindemann, supra, 730 F.2d at 1461, 221 U.S.P.Q. at 488; Stratoflex, Inc. v. Aeroquip Corp., 713 F.2d 1530, 218 U.S.P.Q. (BNA) 871 (Fed. Cir. 1983); Kansas Jack, Inc. v. Kuhn, 719 F.2d 1144, 219 U.S.P.Q. (BNA) 857 (Fed. Cir. 1983); W.L. Gore & Associates v. Garlock, Inc., 721 F.2d 1540, 220 U.S.P.Q. (BNA) 303, 314 (Fed. Cir. 1983).* [**41]

n3 Although the district court failed expressly to find the level of ordinary skill in the art at the time the invention was made, it did make reference to "people working in immunology aware of the Kohler and Milstein discovery" which we deem an accurate finding for the purposes of that portion of the *Graham* factual inquiries.

1. *The Eight Articles "Predicting" Widespread Use of Monoclonal Antibodies*

Before discussing the more pertinent references in this case -- the Oi/Herzenberg and Frankel works -- we cull the other prior art references relied on by the trial court.

First, the latest four of the eight articles that the court stated were of the "utmost importance" because they "predicted" that the breakthrough in production of monoclonal antibodies by Kohler and Milstein would lead to widespread use of monoclonal antibodies in immunoassays are neither 102(a)/103 nor 102(b)/103 prior art because they are dated between late 1979 and March 6, 1980, well after the date of conception and within one [**42] year of the filing date of the '110 patent.

The earliest four of the eight articles, on the other hand, although clearly prior art, discuss *production* of monoclonal antibodies -- admittedly old after Kohler and Milstein showed how to produce them -- but none discloses sandwich assays. At *most*, these articles are invitations to try monoclonal antibodies in immunoassays but do not suggest how that end might be accomplished. To the extent the district court relied upon these references to establish that it would have been *obvious to try* monoclonal antibodies of 10<8> liters/mole affinity in a sandwich immunoassay that detects the presence of or quantitates antigen, the court was in error. *See Jones v. Hardy, 727 F.2d 1524, 1530, 220 U.S.P.Q. (BNA) 1021, 1026 (Fed.Cir. 1984)* ("Obvious to try" is improper consideration in adjudicating obviousness issue). n4

n4 Finding 10, which states that the invention was contemporaneously developed and disclosed in at least five publications and patent applications not listed above *and dated well after the filing date of the '110 patent but before its issuance* is irrelevant for purposes of the hypothesis based on the three factual inquiries required by § 103 as interpreted by *Graham v. John Deere, 383 U.S. 1, 148 U.S.P.Q. (BNA) 459, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966)* because obviousness must be determined as of the time the invention was made. Additionally, they are of little probative value in this case because they are dated December 1981 at the earliest, more than a year after the August 4, 1980, filing date here and roughly two years after conception occurred. Furthermore, simultaneous development may or may not be indicative of obviousness, the latter being the case here for the above reasons and because the other evidence of nonobviousness is adequate, such occurrences having been provided for in *35 USC § 135. Lindemann, supra, 730 F.2d at 1460-61, 221 U.S.P.Q. at 487; Environmental Designs, Ltd. v. Union Oil Co. of California, 713 F.2d 693, 698 n.7, 218 U.S.P.Q. (BNA) 865, 869 n.7 (Fed. Cir. 1983).*

[**43]

2. *The Kohler and Milstein Work, the Cuello Article and the Jeong, Piasio, and Schurr Patents Considered by the Examiner*

The district court's finding that Kohler and Milstein developed a method for producing monoclonal antibodies in vitro is correct, but that finding proves no more; although it made possible all later work in that it paved the way for a supply of monoclonal antibodies, it indisputably does not suggest using monoclonal antibodies in a sandwich assay in accordance with the invention claimed in the '110 patent.

The Cuello reference discloses monoclonal antibodies but not in a sandwich assay. The competitive assay in Cuello, moreover, [*1381] uses only one monoclonal antibody and thus in no way suggests the claimed invention wherein a ternary complex of two monoclonal antibodies and an antigen form a sandwich. Furthermore, the court did not explain how this art, by itself or in combination with any of the other art, suggests the claimed subject matter and thus why that combination would have been obvious. We are of the opinion that it does not.

The district court correctly found that the use of polyclonal antibodies in sandwich assays was well known. [**44] The Jeong patent discloses the use of polyclonal antibodies in a simultaneous sandwich assay, with no suggestion that monoclonal antibodies be so used. It is prior art by virtue of § 102(e), application for the patent having been filed September 5, 1978, its effective date as a reference. The Piasio patent, disclosing a reverse sandwich assay using polyclonal antibodies, and Schurrs, disclosing a forward sandwich assay using the same, both § 102(a) prior art, are likewise devoid of any suggestion that monoclonal antibodies can be used in a similar fashion.

3. *The Oi/Herzenberg Work and the Frankel Article*

Clearly, the most pertinent items of prior art not cited by the examiner are the Oi/Herzenberg work, as described in section A, 3, supra, and the Frankel article. As stated in the discussion of Prior Invention of Another (section III, 2, supra), the Oi/Herzenberg work involved mapping epitopes on a known quantity of antigen. It was not concerned with and does not disclose using monoclonal antibodies of at least 10<8> liters/mole affinity. Oi and Herzenberg testified that they did not know the affinity of the antibodies used, and Ciotti testified that nowhere in that [**45] work is there mention of monoclonal antibody affinity of at least 10<8> liters/mole. On this basis, we conclude that the Oi/Herzenberg work is qualitatively different than the claimed invention; the former is directed to mapping epitopes on a known quantity of antigen and the latter to determine the "presence or concentration of an antigenic substance in a sample of fluid ...." We disagree with Monoclonal that these are "essentially the same thing." Furthermore, it is perfectly clear that this work in no way suggests using monoclonal antibodies of the affinity claimed in the '110 patent. It is because of these differences between the Oi/Herzenberg work and the claimed invention that the fact that an antigen was sandwiched between two monoclonal antibodies in the course of Oi's and Herzenberg's work is not sufficient basis to conclude that the claimed invention would have been obvious at the time it was made to a person of ordinary skill in the art.

Likewise, a conclusion that the invention would have been obvious cannot properly be reached when the Oi/Herzenberg work is considered in view of the Frankel article. Frankel teaches a method for rapid determination of affinity constants [**46] for monoclonal antibodies, some of which clearly have affinities of the order defined by the claims, but does not in any way suggest using two of those antibodies in a sandwich to assay an antigen by forming a ternary complex of labelled antibody, the antigenic substance, and a bound antibody wherein the presence of the antigenic substance is determined by measuring either the amount of labelled antibody bound to a solid carrier or the amount of unreacted labelled antibody. The mere existence of prior art disclosing how to measure the affinity of high affinity monoclonal antibodies is insufficient to support a holding of obviousness. Hybritech's claims define a *process* that *employs* monoclonal antibodies, and does not merely claim antibodies of high affinity. In view of the fact that the Oi/Herzenberg work is not directed to an assay as claimed and does not disclose antibodies of at least 10<8> liters/mole affinity, and further that Frankel fails to suggest using such antibodies in a sandwich assay, the Frankel article does not compensate for the substantial difference between the Oi/Herzenberg work and the claimed subject matter, and therefore those references in combination [**47] cannot support a holding of obviousness.

[*1382] 4. *Objective Evidence of Nonobviousness*

In one part of its opinion the court found that "the commercial success of the kits *may* well be attributed to the business expertise and acumen of the plaintiff's personnel, together with its capital base and marketing abilities" (emphasis ours) and later that "where commercial success is based on the sudden availability of starting materials, in this instance the availability of monoclonal antibodies as a result of the Kohler and Milstein discovery, business acumen, marketing ability, and capital sources, no causal relationship is proven." (Citation omitted.)

i. *Commercial Success: Hybritech's Diagnostic Kits Grabbed a Substantial Market Share*

The undisputed evidence is that Hybritech's diagnostic kits had a substantial market impact. The first diagnostic kit sales occurring in mid-1981, sales increased seven million dollars in just over one year, from $6.9 million in 1983 to an estimated $14.5 million in 1984; sales in 1980 were nonexistent. Competing with products from industry giants such as Abbott Labs, Hoffman LaRoche, Becton-Dickinson, and Baxter-Travenol, Hybritech's [**48] HCG kit became the market leader with roughly twenty-five percent of the market at the expense of market shares of the other

companies. Its PAP kit ranks second only to a product sold by Dupont's New England Nuclear, surpassing products from Baxter-Travenol, Abbott, and others. Hybritech's other kits, indisputably embodying the invention claimed in the '110 patent, obtained similar substantial market positions.

Although the district court did not provide its insights into why commercial success was due to business acumen and not to the merits of the claimed invention, Monoclonal urges in support that it was due to Hybritech's spending disproportionate sums on marketing, 25-30% of income. The undisputed evidence was that expenditures of *mature* companies in this field are between 17 and 32%. Furthermore, the record shows that advertising makes those in the industry -- hospitals, doctors, and clinical laboratories -- aware of the diagnostic kits but does not make these potential users buy them; the products have to work, and there is no evidence that that is not the case here or that the success was not due to the merits of the claimed sandwich assays -- clearly contrary to [**49] the district court's finding.

The trial court's finding that the "sudden availability of monoclonals" was the reason for the commercial success of Hybritech's diagnostic kits (Finding 11) is unsupported by the record and clearly erroneous. Monoclonal admits that monoclonal antibodies were available in the United States in *1978*, and the evidence clearly reflects that. Thus, at least *three years* passed between the time monoclonal antibodies were available in adequate supply and the time Hybritech began selling its kits. Especially in the fast-moving biotechnology field, as the evidence shows, that is anything but sudden availability.

ii. *Unexpected Advantages*

Hybritech points to the testimony of three witnesses skilled in the diagnostic field who state that, based on tests done in their laboratories as a result of real-world comparisons in the normal course of research, the diagnostic kits that embody the '110 invention unexpectedly solved long-standing problems. Dr. Hussa, the head of a large referral laboratory and a world-wide consultant, testified that until Hybritech introduced its kits, he and others were very skeptical and had almost exclusively used competitive [**50] assays with a radioactive tracer (RIAs). n5 In relation to an [*1383] HCG Hybritech kit, he testified that he had first thought that the Hybritech HCG kit would not give accurate results for low antigen concentrations because that condition is indicated in the Hybritech kit by a low radioactivity reading, a reading difficult to differentiate from control samples containing no antigen. He also stated that in the past, RIA kits falsely detected HCG in nonpregnant women, a condition which would indicate cancer and surgery. He stated that when he employed the Hybritech HCG kit in such instances it demonstrated, correctly and absent any difficulty interpreting the data, that no HCG was present.

n5 Monoclonal's expert Blakemore testified that of 425 assays on the market in 1979 less than 1% were sandwich assays. Today, sandwich assays constitute the majority of all assays sold.

The record also shows that Blakemore, who testified extensively for Monoclonal that the claimed invention would have been obvious, never used monoclonal antibodies in sandwich assays at Cetus before 1980. Additionally, she did not even mention them in the Jeong patent, of which she was a coinventor, which issued January 13, 1981, long after the beginning of Hybritech's work in this area in 1979.

[**51]

Dr. Blethen, an M.D. holding a Ph.D. in biochemistry, testified that she did not think that the Hybritech HGH kit, for detecting growth hormone in children, would offer any advantage, but she determined that it detected HGH deficiencies in children where conventional RIAs failed to do so. She also stated that the kit does not give false positive readings as do conventional RIA kits, an opinion shared by Dr. Hussa. A third witness, Dr. Herschman, who holds a master's degree in chemistry, testified that he spent years working on the development of an assay that would determine the presence of TSH (thyroid stimulating hormone) with greater sensitivity. He succeeded but discovered that the Hybritech TSH kit had the same sensitivity, the test being performed in four hours rather than the three days his kit required.

Having considered the evidence of nonobviousness required by § 103 and *Graham, supra,* we hold, as a matter of law, that the claimed subject matter of the '110 patent would not have been obvious to one of ordinary skill in the art at the time the invention was made and therefore reverse the court's judgment to the contrary. The large number of references, [**52] as a whole, relied upon by the district court to show obviousness, about twenty in number, skirt all around but do not as a whole suggest the claimed invention, which they must, to overcome the presumed validity, *Lindemann, 730 F.2d at 1462, 221 U.S.P.Q. at 488, as a whole. See 35 USC § 103; Jones v. Hardy, 727 F.2d 1524, 1529, 220 U.S.P.Q. (BNA) 1021, 1024 (Fed. Cir. 1984).* Focusing on the obviousness of substitutions and differences instead of on the invention as a whole, as the district court did in

frequently describing the claimed invention as the mere substitution of monoclonal for polyclonal antibodies in a sandwich assay, was a legally improper way to simplify the difficult determination of obviousness. *See generally Hodosh v. Block Drug Co., 786 F.2d 1136, 229 U.S.P.Q. (BNA) 182 (Fed. Cir. 1986).* n6

> n6 It bears repeating that it is crucial that counsel set forth the law accurately. More particularly, it is the duty of counsel to impart to the judge that the obviousness question properly is whether the *claimed* invention *as a whole would have been* obvious to one of *ordinary* skill in the art *at the time the invention was made*, and that the district court must *expressly* make the three factual determinations required by *Graham* and consider objective evidence of obviousness *before* the legal conclusion of obviousness vel non is made. Submitting to the court language like "any differences ... would have been obvious," as was done here, violates the axiom that the question is not whether the differences would have been obvious but the claimed invention *as a whole*. Furthermore, arguing that "it would be obvious" rather than that it would *have been* obvious shifts the court's focus to the wrong period of time, namely to a time long after the wrong period of time, namely to a time long after the invention was made, in which, more likely than not, the prior art and the level of ordinary skill in the art are more advanced. *See 35 USC § 103.*

[**53]

With respect to the objective indicia of nonobviousness, while there is evidence that marketing and financing played a role in the success of Hybritech's kits, as they do with any product, it is clear to us on the entire record that the commercial success here was due to the merits of the claimed invention. It cannot be argued on this record that Hybritech's success would have been as great and as prolonged as admittedly it has been if that success were not due to the merits of the invention. The evidence is that these kits compete successfully with numerous others for the trust of persons who have to make fast, accurate, and safe diagnoses. This is not the kind of [*1384] merchandise that can be sold by advertising hyperbole.

V. *Enablement, Best Mode, and Definiteness Under § 112*

The section 112 defense appears to have been an afterthought of both Monoclonal, who briefly but unsuccessfully attempts to defend this utterly baseless determination, and of the district court which adopted the defense from Monoclonal's pretrial papers apparently without knowledge of the applicable law, to highlight, as it stated at trial, that it was part of its job to see that "whoever [**54] wins wins all the way or whoever loses loses all the way." Taken as a whole, the court's comments on § 112 -- split into two parts, one from Monoclonal's pretrial brief and the other from the adopted pretrial findings and conclusions -- are internally inconsistent. The opinion states that the patent fails to disclose how (1) to make monoclonal antibodies; (2) to screen for proper monoclonal antibodies; and (3) to measure monoclonal antibody affinity and therefore the specification is nonenabling and does not satisfy the best mode requirement, and the claims are indefinite. We discuss each of these in turn.

1. *Enablement*

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention, *Raytheon Co. v. Roper Corp., 724 F.2d 951, 960, 220 U.S.P.Q. (BNA) 592, 599 (Fed. Cir. 1983),* is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive, *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co., 750 F.2d 1569, 1576, 224 U.S.P.Q. (BNA) 409, 413 (Fed. Cir. 1984),* [**55] and is determined as of the filing date of the patent application, which was August 4, 1980. *See W. L. Gore and Associates v. Garlock, Inc., 721 F.2d 1540, 1556, 220 U.S.P.Q. (BNA) 303, 315 (Fed. Cir. 1983).* Furthermore, a patent need not teach, and preferably omits, what is well known in the art. *Lindemann, 730 F.2d at 1463, 221 U.S.P.Q. at 489.*

The record fully supports the '110 patent's statement that

> The monoclonal antibodies used for the present invention are obtained by the [hybridoma] process discussed by Milstein and Kohler. ... The details of this process are well known and not repeated here.

The district court itself stated that the "method for producing monoclonal antibodies in vitro was well known prior to the alleged invention of the '110 patent," and used the "sudden availability of monoclonal antibodies" produced by the Kohler and Milstein discovery to support, albeit erroneously, its finding of a lack of nexus between the merits of the claimed invention and its commercial success. The court then about-faced and held the '110 patent deficient because it fails to teach how to make monoclonal antibodies. [**56]

With respect to screening, the only permissible view of the evidence is that screening methods used to identify the necessary characteristics, including affinity, of the monoclonal antibodies used in the invention were known in the art and that the '110 patent contemplated one of those. At trial, Monoclonal's counsel stated "it is a procedure that was known in '78." In similar fashion, the district court held that the claimed subject matter would have been obvious in part because the "existence of monoclonal antibodies *having the affinity constants claimed in the patent was well known* prior to the alleged invention. ..." [Emphasis ours.] Furthermore, there was not a shred of evidence that undue experimentation was required by those skilled in the art to practice the invention. We hold as a matter of law that the '110 patent disclosure is enabling.

2. *Best Mode*

"The specification ... shall set forth the best mode contemplated by the inventor of carrying out his invention." *35 USC § 112.* Because not complying with the best mode requirement amounts [**57] to concealing the preferred mode contemplated by the applicant at the time of filing, in order to find that the best mode requirement is not satisfied, it must be shown that [*1385] the applicant knew of and concealed a better mode than he disclosed. *DeGeorge v. Bernier, 768 F.2d 1318, 1324, 226 U.S.P.Q. (BNA) 758, 763 (Fed. Cir. 1985)* (quoting with approval *In re Sherwood, 613 F.2d 809, 204 U.S.P.Q. (BNA) 537 (CCPA 1980)).* The only evidence even colorably relating to concealment is testimony by various Hybritech employees that sophisticated, competent people perform the screening and that the screening process is labor-intensive and time-consuming. It is not plausible that this evidence amounts to proof of concealment of a best mode for screening or producing monoclonal antibodies for use in the claimed '110 process, and therefore we are of the firm conviction that the district court's finding that the best mode requirement was not satisfied is clearly erroneous.

3. *Indefiniteness*

The basis of the district court's holding that the claims are indefinite is that "they do not disclose how infringement may be avoided because antibody [**58] affinity cannot be estimated with any consistency." (Conclusion 6.) Even if the district court's finding in support of this holding -- that "there is no standard set of experimental conditions which are used to estimate affinities" -- is accurate, under the law pertaining to indefiniteness -- "if the claims, read in light of the specification, reasonably apprise those skilled in the art both of the utilization and scope of the invention, and if the language is as precise as the subject matter permits, the courts can demand no more," *Shatterproof Glass Corp. v. Libbey-Owens Ford Co., 758 F.2d 613, 624, 225 U.S.P.Q. (BNA) 634, 641 (Fed. Cir. 1985)* -- the claims clearly are definite. The evidence of record indisputably shows that calculating affinity was known in the art at the time of filing, and notwithstanding the fact that those calculations are not precise, or "standard," the claims, read in light of the specification, reasonably apprise those skilled in the art and are as precise as the subject matter permits. As a matter of law, no court can demand more.

VI. [**59] *Motions*

Monoclonal's motion to strike Appendices A and B of Hybritech's reply brief as being beyond the page limit applicable to reply briefs is granted as to Appendix A but denied as to Appendix B, the latter having been helpful in culling the often non-supportive citations to the record by Monoclonal.

Hybritech's motion to supplement the record with a Monoclonal advertisement not considered at trial is denied. Any adverse impact that the disposition of these two motions has upon either party is more than outweighed by this court's patience with the seemingly endless flow of post-argument argumentative papers.

VII. *Conclusion*

The judgment of the district court holding the patent in suit invalid is *reversed* in all respects, and the case is *remanded* for a determination of the issue of infringement which the court held was moot.

REVERSED AND REMANDED.